1955

Fractionation of Pituitary Tissue by Differential Centrifugation

Daniel Martin Ziegler
Loyola University Chicago

FRACTIONATION OF PITUITARY TISSUE BY DIFFERENTIAL CENTRIFUGATION

by

Daniel Martin Ziegler

A Thesis Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

June

1955

LIFE

Daniel Martin Ziegler was born in Quinter, Kansas, July 6, 1927.

He was graduated from Quinter High School in May, 1944. In June, 1949, he was graduated with the degree of Bachelor of Science, from St. Benedict's College, Atchison, Kansas. He received the Master of Science degree in June, 1952, from the Institutum Divi Thomae, Cincinnati, Ohio.

He began further graduate studies at Loyola University in September, 1952. From June, 1954, he held the Royal E. Cabell Fellowship.

Portions of this work were presented in March, 1955, at the national meeting of the American Chemical Society, at Cincinnati, Ohio.

ACKNOWLEDGEMENTS

The author wishes to express his gratitude to Dr. Jacklyn B. Melchior, for her help and encouragement throughout this work.

He also wishes to thank Dr. Arthur J. Gatz for his aid in the cytological examinations of the fractions, and Dr. Leo Abood, of the University of Illinois, for the use of the Spinco ultracentrifuge. Wilson Laboratories were also most generous in their gift of certain of the experimental animals.

TABLE OF CONTENTS

TABLE OF CONTENTS (Cont'd)

LIST OF FIGURES

LIST OF TABLES

LIST OF TABLES (Cont'd)

CHAPTER I

INTRODUCTION

Statement of the Problem.

The study of protein synthesis has received great impetus since the advent of suitable radioactive isotopes. Protein synthesis on the cellular level cannot be adequately studied in the intact animal, and in *in vitro* systems, proteolysis usually exceeds synthesis, resulting in a net decrease in protein. However, with C^{14} or S^{35} labeled amino acids, protein synthesis can still be studied in these preparations, since newly formed protein is automatically labeled, even while the rate of proteolysis exceeds that of synthesis. The rate of incorporation of the labeled amino acids may therefore be used as a measure of the protein synthesizing enzymes.

It has been reported (Melchior and Halikis 1952) that *in vitro* preparations of pituitary were particularly active in the protein labeling enzymes. In addition, it was shown that the activity of these enzymes was significantly altered by physiological changes known to be related to pituitary function, which suggests that the rate of incorporation of labeled amino acids by this tissue may be related to synthesis of the protein hormones.

In the work presented here, studies were made on the location of the protein hormones, and the *in vitro* labeled protein in the pituitary cell. After the *in vitro* labeling reaction and homogenization of the

tissue, separation of the cellular components was accomplished by differential centrifugation. Studies were made on pituitary tissue from both normal and diethylstilbesterol treated animals. Diethylstilbesterol is known to interfere with the normal physiological function of the pituitary.

For purposes of comparison, similar labeling studies were conducted on liver, since this tissue is also known to be active in the production and secretion of protein. Furthermore, the particulate components of this tissue, isolated by differential centrifugation, have been well characterized (Schneider and Hogeboom 1951). In the course of the work with liver tissue a striking activation of the protein synthesizing enzymes was observed to occur during incubation. Some investigations on the nature of this effect are also included in the work presented here.

Protein Hormones of the Pituitary Gland.

The pituitary (hypophysis cerebri), a small gland at the base of the brain, regulates many of the endocrine glands by its secretion of protein hormones. Structurally, it consists of two main parts; the posterior pituitary, and the larger, anterior pituitary.

The hypophyseal protein hormones are believed to be excreted exclusively by the anterior lobe. Histologically, three types of cells can be demonstrated in this part of the gland. In the adult rat the alpha cells represent about 40-45 per cent of the total epithelial cells, and contain granules which stain with acid dyes. The basophilic cells make up about 5-7 per cent of the total cells, and their granules stain with basic dyes. The remaining approximately 50 per cent of the epithelial

cells are chromophobes which are not stained in ordinary preparations.

The pituitary produces several protein hormones, six of which have been well characterized and highly purified. The presence of the gonadotropic hormones was definitely established by Smith and Engel (1927) and Zondek and Aschelm (1927). Hypophyseal gonadotropin consists of at least two distinct hormones; (1) a follicle stimulatory hormone, which produces hypertrophy of the follicles in the ovary; (2) a hormone responsible for the corpus luteum development and stimulatory to the interstitial testicular tissue -- the luteinising or interstitial cell stimulating hormone. At least one of the gonadotropins is believed to be elaborated by the basophilic cells of the anterior hypophysis, since castration produces a marked hypertrophy of the anterior lobe basophils, along with an increase in the gonadotropin content of the pituitary.

The presence of a growth stimulating factor in the anterior hypophysis was noted by Evans and Long (1921). Smith (1930) demonstrated that hypophysectomized rats ceased growing, but a resumption of growth occurred upon implantation of whole pituitary glands. The growth hormone has been highly purified (Li et al. 1944, 1945). It is generally agreed (Selye 1949) that growth hormone is secreted by the eosinophils, as eosinophil adenomas are commonly associated with acromegaly, and in some animals the relative number of eosinophils is greatest during maximal growth.

Adrenocorticotropin activity of the pituitary was first described by Smith (1930). This hormone, recently isolated in a very pure form (Li et al. 1942; Sayers et al. 1943), can produce hypertrophy of the adrenal cortex, but has no apparent effect on the medulla. The

adrenocorticotropic hormone appears to be elaborated chiefly by the basophils, since basophilic adenomas are accompanied by proliferation of adreno-cortical tissue and an increased corticotropin production (Selye 1949).

The regulation of the thyroid by a protein hormone of the pituitary has been well established (Smith 1927, 1930; Loeb and Bassett 1929, 1930). This thyrotropic hormone has been prepared from bovine pituitaries free from the other pituitary hormones (White 1944).

The presence of the lactogenic hormone in the anterior pituitary was first demonstrated by Stricker and Grueter (1928). This hormone was the first of the pituitary protein hormones to be isolated in a chemically pure form (White et al. 1937). Lyons (1942) demonstrated that the lactogenic hormone could initiate lactation by a direct action on mammary tissue. This hormone is probably secreted by the acidophil cells (Smelser 1944).

It might be expected that pituitary tissue would be very active in the incorporation of labeled amino acids, since it is so highly specialized in the production of protein hormones. This was found to be the case (Melchior and Halikis 1952) in studies on the in vitro incorporation of S^{35}- methionine by rat pituitary tissue. The protein synthesizing enzymes of pituitary were reported to be two to three times as active as those of liver tissue. In addition, these investigators demonstrated that the activity of the protein synthesizing enzymes was significantly altered by physiological changes known to be related to pituitary function.

The methionine incorporating activity of glands from very young

animals (10-15 gms.) was approximately 33 per cent greater than that of older animals. After the first two weeks, the activity of the glands remained relatively constant throughout life, with some tendency to decrease in older rats. It is interesting to note that the growth rate of rats has been shown to be greatest during the first few days of life (Donaldson 1924), and it would seem during this period, the pituitary, which is known to have a high percentage of eosinophilic cells (Selye 1949), is primarily secreting growth hormone.

The state of activity of the pituitary during lactation has been studied by Hurst and Turner (1942). They report an increased rate of lactogenic hormone production during the first three days after parturition, and then a slow diminishing of the rate. Melchior and Halikis (1952) found that seven days after parturition the protein synthesizing enzyme activity of the pituitary was significantly less than that of a non-lactating female. Since the estrus cycle is halted during lactation, it would certainly suggest that the incorporation of labeled methionine is related to the synthesis of protein hormones.

Relationship of in Vitro Amino Acid Incorporation to Physiological Protein Synthesis.

With the aid of methionine and cystine labeled with S^{35}, Melchior and Tarver (1947a, 1947b) were the first to demonstrate that protein synthesis could occur in tissue slices in an artificial medium. They incubated rat liver slices with S^{35} methionine in Ringer's bicarbonate solution, and found that a portion of the labeled amino acid was incorporated into the

protein of the tissue. Following this work it was rapidly established that a variety of surviving tissue preparations could incorporate labeled amino acids into protein. Studies were made on bone marrow cells (Borsook et al. 1950b), intestinal sections (Winnick et al. 1947), embryonic tissue (Zamecnik et al. 1948), and tumors (Farber et al. 1951; Zamecnik et al. 1948; Kit and Greenberg 1951). The rates of incorporation of the amino acids by these systems were found to be of the same order of magnitude observed in vivo (Borsook 1950). Liver homogenates were also shown to incorporate labeled amino acids (Melchior and Tarver 1947a), but the rate was much lower than in slices.

The basic assumption in this in vitro incorporation of labeled amino acids is that the uptake is actually due to peptide bond formation, that the label is not simply adsorbed on the protein, or bound by other than peptide bonds. Considerable evidence has been accumulated in the literature which indicates that the incorporation does represent peptide bond formation. It has been demonstrated (Winnick 1950a, 1950b; Peterson and Greenberg 1952) that with washed protein preparations from incubations with C^{14}-carboxyl labeled amino acids, little or none of the radioactivity can be liberated by treatment with ninhydrin. The ninhydrin reagent liberates CO_2 from the carboxyl group of amino acids only when both the carboxyl and amino groups are free. However when the labeled protein is successively hydrolyzed by a series of proteolytic enzymes (pepsin, trypsin, carboxypeptidase), the amount of radioactivity released by ninhydrin progressively increases (Winnick et al. 1949; Peterson and Greenberg 1952). Prolonged dialysis of the in vitro labeled protein

dissolved in basic urea resulted in only a slight decrease in the radioactivity of the protein (Simpson and Tarver 1950; Kit and Greenberg 1952).

The question then arises whether the incorporation of amino acids by surviving tissue has any relation to physiological protein synthesis in the intact animal, or if it merely represents non-enzymatic exchange or reversal of proteolysis in the tissue. Non-enzymatic exchange can be ruled out, as it has been repeatedly shown that the integrity of the enzymes is essential for incorporation. Cytolysis of the tissue by freezing and thawing, lysis in water, or lyophilization completely abolishes the uptake of amino acids by the tissue (Borsook et al. 1950b; Winnick et al. 1948). Liver slices kept at 0° - 5°C for two hours suffer a great diminution in their ability to incorporate labeled methionine (Simpson and Tarver 1950). This not only indicates that incorporation occurs through the mediation of enzymes, but that organization of the protein synthesizing enzymes in the cell is essential for activity.

One apparent exception is the anomalous incorporation of glycine, alanine, phenylalanine, and lysine into the desoxypentose nucleohistones prepared from rat liver (Brunich and Luck 1952). The uptake of labeled amino acids by this preparation was linear with time, and the rate increased with increases in temperature -- up to 100°C. The nature of the bond formed between the amino acid and the histone is not clear, but it is evident that the reaction is non-enzymatic. However the rate of incorporation by such preparations is much lower than in tissue slices or even homogenates. It would appear that this mechanism is a minor one in the overall incorporation of amino acids by liver preparations.

Other non-enzymatic incorporation has been shown to occur in certain instances. It has been demonstrated that the cystine taken up by liver preparations could largely be removed by treating the protein with reducing agents such as 2-mercaptoethanol (Melchior and Tarver 1947a; Peterson et al. 1951). The incorporation in this case was presumably due to disulphide bond formation. Therefore, when using cystine, or any labeled amino acid that could form appreciable amounts of radioactive cystine, it is necessary to remove this non-peptide bound label before measuring the radioactivity of the protein. Glycine may also be incorporated by disulphide bond formation through glutathione in liver homogenates, since this tissue is very active in the synthesis of this peptide (Johnson and Bloch 1951). Thirty to 70 per cent of the glycine incorporated by liver homogenates can be removed by reduction of the labeled protein (Peterson and Greenberg 1952), so it would appear that a large part of the incorporated glycine is bound through disulphide bonds.

It has been reported that the free energy of formation of the peptide bond in a number of simple di- and tri- peptides is around +3000 calories (Borsook and Dubnoff 1940). Direct measurements on the free energy of formation of peptide bonds in proteins are not available, but it has been suggested (Linderstrøm-Lang 1952) that the greater separation of charges on long peptides would reduce the energy required to add another amino acid to the chain. Even when this factor is taken into consideration, the net free energy of formation of proteins must be positive, since spontaneous hydrolysis of the protein does occur in the absence of an added energy source. Thus it is clear that over the physiological pH range, and

at any practical amino acid concentration, the equilibrium is far on the side of hydrolysis of the protein. However this equilibrium is a dynamic one, and in the presence of suitable proteolytic enzymes, it is conceivable that a small amount of labeled amino acid could be incorporated into protein by exchange in this manner. Incorporation by this process would not, of course, result in net synthesis of protein, and would not have to be coupled with any energy donating system.

There is abundant evidence that incorporation in in vitro preparations does not occur by this mechanism, but that the labeling reaction must be coupled with an energy donating system. Inhibition of respiration or interference with phosphorylation abolishes the incorporation of amino acids into protein. Thus it has been shown that the uptake of methionine by non-proliferating E. coli was inhibited by azide, fluoride, and cyanide (Melchior et al. 1948); anaerobiosis and dinitrophenol inhibited the incorporation of alanine by rat liver slices (Frantz et al. 1947, 1948). In liver homogenates fluoride, arsenate, methylene blue, toluene, and n-octanol practically abolished incorporation of glycine (Peterson and Greenberg 1952).

An exception is the enzymatic incorporation of L-lysine by guinea pig liver homogenates (Borsook et al. 1949, 1950a; Schwett and Borsook 1953). The L-isomer, but not the D form, is readily incorporated under anaerobic conditions by the whole homogenate. This system has another peculiarity in that the optimum pH is 6.1. A particulate sediment obtained from these homogenates by centrifugation at 2500xg can also incorporate L-lysine but this system requires oxygen for maximum uptake of

the label. However the rate of incorporation is not affected by fluoride, azide, or dinitrophenol, and is independent of the concentration of labeled lysine but depends only on the total amount of lysine in the system. These lysine incorporating systems of liver apparently belong in a class by themselves, as they appear to be characteristically different from the other *in vitro* protein labeling preparations referred to previously.

The most convincing evidence that *in vitro* uptake of amino acid is a measure of a physiological process of protein synthesis is offered by some studies on the incorporation of the label into specific proteins. It has been shown that chicken liver slices incorporate amino acids into serum albumin from C^{14} bicarbonate in the medium (Peters and Anfinsen 1950a); slices of liver and spleen from immunized rabbits were shown to incorporate labeled glycine into antibodies to Type III pneumococcus (Ranney and London 1951). Hokin (1951a, 1951b, 1952) demonstrated that pancreatic tissue slices from pigeons could synthesize pancreatic amylase, and Dhungar and Sreenivasan (1952) have shown that liver slices from protein depleted rats were able to synthesize xanthine oxidase *in vitro*.

The work of Peters and Anfinsen and that of Hokin will be considered here in detail, as in both cases there occurred a net synthesis of a specific protein *in vitro*.

In the experiments of Hokin the pancreatic slices were treated with carbamylcholine to deplete the tissue of as much as possible of the preformed enzyme and zymogen, and then incubated in Ringer's bicarbonate supplemented with a casein hydrosylate and glucose. The amylase was determined by a standard method before and after incubation. During the

course of incubation the amylase activity was observed to increase at an average rate equivalent to five mg. of amylase per gram of dry weight of tissue per hour. There was no increase in enzyme activity when the tissue was incubated anaerobically or in the presence of cyanide, dinitrophenol, or iodoacetate. This shows that the increase in amylase during incubation was not due to formation of the enzyme from zymogen initially present in the tissue.

In the studies of Peters and Anfinsen, liver slices from chicks were washed for one hour in Ringer's bicarbonate in order to lower the initial level of serum albumin. The tissue was transferred to a new medium containing C^{14} bicarbonate. It had previously been shown that $C^{14}O_2$ was readily incorporated into amino acids (Anfinsen et al. 1949). After two to four hours under 95% O_2 - 5% CO_2 at 38 C. the tissue and medium were subjected to an ethyl alcohol fractionation. The fraction with the highest specific activity was obtained at 33-42% alcohol. Electrophoretic mobility and the solubility characteristics of the proteins in the fraction indicated that it was serum albumin. The bulk of the radioactive protein of this fraction could be precipitated with a specific antibody prepared from rabbits injected with chicken serum albumin.

In a later study Peters and Anfinsen (1950b) were able to show that the amount of albumin precipitated by the antibody increased during the course of incubation, and after an initial lag of thirty minutes, the increase in serum albumin was linear with time, up to four hours. The average net synthesis was 0.12 mg. serum albumin per gram liver tissue per

hour. This rate was somewhat lower than that calculated from *in vivo* determinations on rats and dogs (Tarver 1954). However, a slower rate might be expected in tissue slices, as the proportion of functioning cells cannot be accurately determined. Sectioning damages some of the cells of the periphery, and the metabolism of the cells in the center of the slices may be limited by diffusion of metabolites from the medium.

Fractionation of Tissue by Differential Centrifugation.

The method of differential centrifugation of broken cell suspensions was introduced by Bensely and Hoerr (1934), who were able to obtain a reasonably pure preparation of mitochondria from guinea pig liver. Following the publication of this paper, the technique of differential centrifugation was employed to obtain preparations of nuclei (Claude 1943a, 1943b), and chromosomes (Claude and Potter 1943; Mirsky and Pollister 1943; Mirsky and Ris 1947a, 1947b). In most of the early work the tissue was fractionated in electrolyte solutions, physiological saline or Ringer's solution being the most commonly employed. The one exception is the method of Behrens (1932), in which the tissue was first lyophilised, then ground to disrupt the cells, suspended in organic solvents, and fractionated by differential centrifugation. By this method good yields of nuclei were obtained, but they showed extensive morphological alterations (Dounce *et al.* 1950). Nuclei and cytoplasmic particles isolated in media containing electrolytes appeared to undergo morphological changes after disruption of the cell. Furthermore, the presence of electrolytes produced pronounced aggregation of the cytoplasmic particles, making

it impossible to obtain a good separation of these particles and the nuclei.

The use of hypertonic solutions of non-electrolytes for the dispersion of cell components was first described by Hogeboom, Schneider, and Pallade (1948). They disrupted the cells of rat liver tissue in 0.88 M sucrose, and by using increasing centrifugal forces separated the tissue into four fractions. First, a nuclear fraction containing about fifteen per cent of the original total nitrogen, and including all of the nuclei of the homogenate, together with some residual intact cells and a variable but usually small number of free mitochondria; second, a large granule fraction, in which practically all of the visible particles satisfy the morphological and cytological criteria of mitochondria. The third fraction, the small granule or microsome fraction, contained 20 to 25 per cent of the original nitrogen. The fourth fraction, the final supernate, contained 30 to 40 per cent of the original homogenate nitrogen, and consisted of the soluble material of the cell. A similar procedure using isotonic sucrose (0.25 M) was later published by Schneider (1948). In isotonic sucrose the characteristics of the fractions obtained were essentially the same as those from hypertonic sucrose, except that in the isotonic medium the mitochondria tended to lose their elongated shape, and become spherical after a few hours.

Enzymes and Nucleic Acids Associated with Cellular Structures. The fractionation of tissue by differential centrifugation has been the subject of two excellent reviews (Schneider and Hogeboom 1951; Hogeboom 1951). The optimum conditions for obtaining adequate separation of the

components is discussed in great detail in these reviews, and the criteria for identifying a biochemical entity with a particulate structure is also given. Briefly, these criteria may be listed as follows: (1) the fraction must be homogenous and the particulate element must not show extensive morphological or cytological changes in the isolated form; (2) the concentration of the substance must be greater in this fraction than in the whole homogenate; (3) the concentration of the substance must not be decreased appreciably by repeated washing of the particles.

On the basis of the above criteria several enzymes and the nucleic acids have been shown to be associated with particulate elements in liver cells. Practically all of the desoxyribose nucleic acid was associated with the nuclei (Hogeboom et al. 1948); the bulk of the succinoxidase activity and cytochrome C was found in the mitochondria fraction (Schneider et al. 1948; Schneider and Hogeboom 1950). The highest concentration of ribosenucleic acid was found in the microsome (small granule) fraction (Hogeboom et al. 1948; Schneider 1948; Schneider et al. 1950). However, a considerable portion of this nucleic acid was found in the soluble fraction, and not all of it could be due to unsedimented particles.

Protein Labeling Studies. The technique of differential centrifugation has been used to study the turnover rate of proteins in the particulate components of liver tissue. Hulten (1950), by injecting young chicks with radioactive glycine, and also Borsook et al. (1950d), by injecting guinea pigs with radioactive glycine, histidine, leucine, and lysine, found that the highest rate of incorporation of the labeled amino

acids was in the microsome fraction. Keller (1951) reported similar results after injecting rats with radioactive leucine.

Rat liver homogenates incubated with C^{14} alanine and then fractionated by differential centrifugation in sucrose solution also exhibited the highest rate of incorporation in the microsome fraction (Siekewitz 1952). The lowest rate of incorporation of the labeled alanine occurred in the soluble fraction. The rate of label uptake by the nuclear and large granule fractions is difficult to assess, since agglutination of the particles undoubtedly occurred during incubation, and these fractions were probably contaminated with microsomes.

Fractionation of Pituitary by Differential Centrifugation. A survey of the literature indicates that only a few attempts have been made to separate the hormones of the anterior pituitary by differential centrifugation. There were no reports where distinct separation of the hormones was obtained, and in each case the method of homogenation or fractionation employed was different from those shown to be necessary to obtain good separation of the particulate components of liver tissue. The reports by three different investigators on the sedimentation characteristics of the granules containing the gonadotropin hormones show conflicting results. This may be partially attributed to the different methods of fractionation used by these workers.

The first work on the distribution of hormones in pituitary granules obtained by differential centrifugation was that of Catchpole (1948). The fractionation was carried out in isotonic saline, and the fractions were assayed for gonadotropin activity. This hormone was found

in a large granule fraction and the soluble fraction. The large granule fraction was not further characterized, but from the size of the granules, it was assumed to contain the mitochondria of the tissue.

In 1949 McShan and Meyer were able to obtain a partial concentration of the gonadotropic hormone in granule fractions obtained from normal and castrated rats, by a modification of the method of Hogeboom et al. (1948). The tissue was homogenized in a cone homogenizer in 0.88 M sucrose, and then subjected to centrifugal forces ordinarily used in fractionations carried out in 0.25 M sucrose (Schneider 1948). The fractions were assayed on immature rats of the Holtzman strain, and the increase in ovarian weight was used to measure the gonadotropin hormones. No data was reported as to the relative number of corpora lutea or of follicles in the ovaries.

Increases in ovarian weight occurred in animals receiving injections of the large granule, small granule, and the supernate fractions, with the greatest effect produced by the small (15,000xg for one hour) granule fraction. This was true of tissue from both normal and castrated rats. The authors state that 60 per cent of the succinoxidase activity was recovered in the large granule fraction.

In later studies Meyer and McShan (1952; McShan and Meyer 1953) were able to reduce the amount of gonadotropin in the supernate fraction by disrupting the cells by mashing instead of homogenizing. In these papers they report that 75 per cent of the gonadotropin activity was recovered in a granule fraction obtained by centrifuging at 20,400xg for one hour. However, an examination of their data shows that they obtained this

concentration of gonadotropin only with pituitary from castrated rats. The distribution of protein and succinoxidase differs radically from that reported for normal liver (Hogeboom et al. 1948). It is difficult to correlate their observation, that the mitochondria were sedimented at 4,400xg for twenty minutes, with the forces necessary to sediment mitochondria of liver, 24,000xg for twenty minutes, in the same medium. It has been shown (Kennedy and Lehninger 1949) that electrolytes must be added to the medium to sediment mitochondria from rat liver at the low force used by McShan and Meyer. Examination of their data shows that approximately 30 per cent of the total protein and 50 per cent of the succinoxidase activity were recovered in the nuclear fraction. That the large percentage of succinoxidase and protein recovered in this fraction is not due to the presence of whole cells is indicated by the absence of the gonadotropins in the fraction. From the low centrifugal forces they found necessary to sediment the granule fractions, it would appear that agglutination of the particles occurred in the dispersion medium, possibly by the contamination of the sucrose with electrolytes.

Other attempts to fractionate pituitary tissue by differential centrifugation have been reported by Herlant (1952). In the work of this investigator, pituitary tissue, usually from sheep, was fractionated in 0.44 M sucrose into three fractions. A nuclear fraction, which also contained the cellular debris; a granule fraction, which was stated to consist mainly of eosinophilic granules; and the supernatant material that remained after sedimentation of the granule fraction. The granule and the supernate fractions were assayed for gonadotropin on immature female rats, and only

the supernatant material gave a significant increase in ovarian weight. The injection of the granule fraction into rats was observed to produce a pronounced decrease in the eosinophilic lymphocytes in the blood. This was taken as evidence of the presence of adrenocorticotropin in this fraction, since Thorn *et al.* (1948) has shown that the eosinophil count falls following the administration of this hormone.

CHAPTER II

EXPERIMENTAL

Animals.

Albino rats of the Sprague-Dawley strain were used throughout this study. The normal animals were raised in the Loyola colony or were purchased from the Hormone Assay Company, and were fed ad libitum up to the time of sacrifice.

Immature rats, hypophysectomized by the Hormone Assay Company, Chicago, were used for all of the hormone assays conducted in this work. These animals were maintained on Astwood diet (Melchior and Sliwinski 1954; see Table I for composition) and were fed ad libitum up to the time of sacrifice.

Tissue Labeling.

The animals were killed by a sharp blow on the back of the neck, and the tissue to be used was removed within one or two minutes after the death of the animal. The pituitaries were used whole or were cut in half with the edge of a spatula. The liver was sectioned approximately 0.5 millimeters thick with a mechanical slicer, constructed in our laboratory, and similar in principle to the Stadie slicer (Umbreit et al. 1949).

The tissue was weighed on a Roller-Smith torsion balance and then placed in the center chamber of a Warburg flask containing the incubation medium. The medium was Ringer's bicarbonate buffer (Melchior 1946), supplemented with 7.4×10^{-3} M sodium succinate. The final pH was

TABLE I

COMPOSITION OF ASTWOOD DIET

Material	Amount (Grams)
Casein	6250
Milk Powder	2500
Dried yeast	2500
Desiccated liver	750
Salt mixture*	500
Sucrose	11250
Corn oil	1250

*This item was obtained from General Biochemicals Inc., Ohio, and was stated to have the following composition (per cent by weight). $CaCO_3$ 54.3, $MgSO_4$ 1.6, NaCl 6.9, KCl 11.2, KH_2PO_4 21.2, $FePO_4$ 2.0, KI 0.008, $Na_2(SO_4)_3$ 0.035, NaF 0.10, $KAl(SO_4)2$ 0.017, $CuSO_4$ 0.09, $MgCO_3$ 2.5.

always 7.4. In a few of the liver labeling experiments rat blood serum was used as the incubation medium.

The side arm of the flask contained one micromole of S^{35} labeled methionine dissolved in 0.5 milliliters of Ringer's bicarbonate solution. The labeled methionine was obtained from Dr. D. L. Tabern of the Abbott Laboratories, and had a specific activity of approximately seventeen microcuries per gram.

The flasks were placed in a water bath maintained at 37.5°C, and were shaken at one hundred twenty revolutions per minute. Unless otherwise specified, the flasks were filled with 95% O_2 - 5% CO_2 by evacuating three times with a vacuum pump. The labeling reaction was started by transferring the methionine from the sidearm into the main chamber of the Warburg flask. The final concentration of the methionine was always one half micromole per milliliter, usually in two milliliters.

Fractionation.

At the end of the incubation period the contents of the flasks were pooled and transferred to a centrifuge cone kept in an ice bath. This material amounted to approximately 120 to 200 milligrams of tissue in each experiment. The tissue was removed from the medium by centrifuging at 600xg for ten minutes. The supernate was decanted and the tissue washed once with two milliliters of 0.25 M sucrose. The wash was combined with the supernate and designated the medium fraction. This fraction contained all the protein that diffused into the medium during incubation.

The washed tissue was homogenized in 1.5 milliliters 0.25 M sucrose, using an homogenizer essentially the same as that described by

Potter and Elvehjem (1936), except that the pestle was made of teflon instead of glass. The homogenizing tube was surrounded by an ice jacket, and the temperature of the tissue suspension did not rise above 5°C during the homogenization. The pestle was driven at approximately one thousand revolutions per minute, and the tissue was homogenized at this speed for three minutes.

The homogenate was transferred to a cold twelve milliliter centrifuge tube with the aid of 0.5 milliliters of the sucrose solution, and was centrifuged at 600xg for ten minutes in a refrigerated centrifuge set at 1°C. The supernatant suspension was carefully decanted and the sediment was washed twice by rehomogenizing in 1.5 milliliters sucrose and centrifuging at the same speed and for the same length of time as before. The washed sediment constituted the nuclear fraction.

The washes and the supernatant material, remaining after the sedimentation of the nuclei, were combined and centrifuged at 8,500xg for twenty minutes in a PR-1 International Refrigerated Centrifuge fitted with a multispeed head. The resulting supernate was decanted and the residue was resuspended in 1.5 milliliters sucrose solution with the aid of a glass homogenizer pestle, constructed to fit the plastic centrifuge tubes. The suspended granules were centrifuged at 8,500xg for ten minutes. The washed residue was designated the large granule fraction.

The small granule fraction was sedimented by centrifuging the combined supernates, obtained in the previous step, at 24,000xg for three hours. The resulting supernate was decanted and constituted the supernate fraction. In one experiment the small granules were sedimented at 110,000xg

for thirty minutes in a Spinco Refrigerated Centrifuge. This centrifugal force was sufficient to sediment particles larger than fifty millimicrons in diameter.

Washing and Counting.

As soon as the fractions were obtained they were suspended in 0.33 M (5%) trichloroacetic acid. In those experiments where the tissue was not fractionated, it was suspended in the TCA at the end of the incubation period and then homogenized. In either case, the samples were subjected to the washing procedure of Melchior and Halikis (1952).

In this procedure the TCA precipitated proteins were washed twice with 5% TCA at the centrifuge, and then dissolved in ten milliliters of 0.1 N base. The base was neutralized with 2N HCl, and the protein reprecipitated by adding ten milliliters of 10% TCA. After one hour at room temperature, the samples were centrifuged, and the supernates discarded. The protein was dissolved and reprecipitated a second time to insure removal of the physically adsorbed radioactivity. The washed protein samples were hydrolyzed in a hydrochloric acid - formic acid solution (Miller and Du Vigneaud 1937), by refluxing under air condensers at 100°C - 108°C for thirteen hours.

The hydrolysates were evaporated to dryness on a steam bath and any labeled mercaptans present were then removed by the method of Zittle and O'Dell (1941). This method removes cystine, cysteine, and homocystine, by precipitation of their insoluble cuprous salts, leaving the methionine in solution. Inactive cystine (2.4 mgs. per sample) was added to serve as

a carrier before the addition of the cuprous oxide.

The excess cuprous oxide and the insoluble cuprous mercaptides were removed by centrifugation. The supernatant liquid was decanted and the residue washed once. The combined supernate and wash of each sample were evaporated to dryness on a steam bath, and the resulting residues transferred quantitatively with the aid of 0.1 N HCl to plastic planchets one and one-fourth inches in diameter for counting. The bottoms of the planchets were completely covered with Whatman No. 1 filer paper. The planchets were transferred to a forced air dryer, constructed so that the air was continuously recirculated over the planchets and a desiccant, which consisted of sodium hydroxide flakes and anhydrous calcium chloride.

Four hours was usually the time required to dry the samples, after which time their radioactivity was measured in an internal counting tube of a proportional counter. This method of preparing and counting samples is essentially the same as that reported by Melchior and Goldkamp (1954).

To check the efficiency of the washing procedure, "zero time" incorporation (Simpson and Tarver 1950) of the label was determined. In these experiments the tissue was incubated for three to six minutes without the label. Immediately after the addition of the label, the material was fractionated and prepared for counting in the manner described above. The label present in the fractions was believed to be a measure of the methionine physically adsorbed on the protein. The "zero time" uptake in both liver and pituitary tissue is shown in Table II, and was zero or negligible in all except the medium fractions. In the data given in

TABLE II

ZERO TIME INCORPORATION

Fraction	Micromoles S^{35}- methionine incorporated per gm. protein N	
	Pituitary[1]	Liver[2]
Nuclei	0.05 ± 0.01	0.0
Large granules	0.03 ± 0.01	0.0
Small granules	0.0	0.0
Supernate	0.04 ± 0.01	0.0
Medium	1.35 ± 0.13	2.7, 2.6

1). The average of three determinations ± the standard error of the mean.

2). Two determinations.

Chapter III the incorporation of the label into the medium fraction was corrected for the zero time uptake.

Nitrogen Determinations.

An aliquot of each sample was removed, usually during the washing procedure when the protein was dissolved in base, for the nitrogen determination. The method employed for the nitrogen analysis was a modification of the procedure of Thompson and Morrison (1951).

The aliquots were heated with 1.5 milliliters of 4.5 M H_2SO_4 on a sand bath for twenty minutes. After clearing with 30% hydrogen peroxide, the digested material was quantitatively transferred to a volumetric flask, the acid partially neutralized with three milliliters of 2 N NaOH, and then diluted to volume with water. The final volume was generally twenty-five milliliters, but in a few experiments where the amount of protein was small, the digests were transferred to five or ten milliliter flasks. An aliquot of the diluted sample was removed and the acidity determined by titration with 0.5 N sodium hydroxide. Then aliquots of the digest (usually one, two and four milliliters) were transferred to colorimeter tubes, calibrated at the ten milliliter mark.

Each tube was diluted to approximately nine milliliters with water and the amount of standard base necessary to neutralize all but 0.15 milliequivalents of the acid. Three tenths of a milliliter of Nessler's reagent, prepared according to the method of Bock and Benedict (Hawk and Bergeim 1937), was added; then the tube was immediately diluted to volume and mixed. The color was permitted to develop at room temperature for

exactly ten minutes before reading in a Klett-Summerson colorimeter with a blue filter. The nitrogen content of each tube was read directly from a standard curve (Figure I) determined with an ammonium sulphate solution of known concentration. The total nitrogen of each sample was then calculated.

Determination of Succinoxidase and Nucleic Acids.

The succinoxidase activity was determined by the method of Schneider and Potter (1943). The details of the method were given in Manometric Techniques and Tissue Metabolism (Umbreit et al. 1949). The fractions were always assayed at two concentrations.

The Warburg flasks, containing the materials listed in Table III, were incubated at 37°C and readings were taken every ten minutes for ninety minutes. The $Q_{O_2(N)}$ was calculated for each flask and the values obtained for each fraction were averaged.

The small granule and supernate fractions were always assayed together, since considerable inactivation of the enzyme occurred during the three hours required to sediment the small granules. The succinoxidase determinations were usually completed three to six hours after the tissue was removed from the animals.

The nucleic acids were extracted by the method of Schneider (1945) from the proteins precipitated with TCA. Briefly, the procedure is as follows; the precipitated protein was washed with 5% TCA three times to remove traces of sucrose and soluble phosphate. The washed residue was suspended in 95% ethyl alcohol and centrifuged; the phospholipides were

TABLE III

CONTENTS OF FLASKS IN SUCCINOXIDASE DETERMINATIONS

Materials	Flasks	
	1	2
0.1 M phosphate buffer pH 7.4	0.6 ml.	0.6 ml.
0.5 M Na succinate pH 7.4	0.2	0.2
$1x10^{-4}$ M cytochrome C	0.3	0.3
$4x10^{-3}$ M $CaCl_2$	0.2	0.2
$4x10^{-3}$ M $AlCl_3$	0.2	0.2
Fractions suspended in 0.25 M sucrose	0.3	0.5
0.25 M sucrose (to make 2.0 ml.)	0.2	0.0

The center wells contained 0.2 ml. of 20% KOH with 3 $cm.^2$ folded filter paper.

removed from the residue by heating three times for three minutes each, with five milliliter portions of a solution containing three parts ethanol and one part diethyl ether. The extracts were discarded and the residue suspended in 5% TCA, heated for fifteen minutes at 90°C, cooled and centrifuged. The precipitate was washed once with 5% TCA and the wash liquid was combined with the TCA extract. This solution contained practically all of the nucleic acids of the tissue.

The desoxyribose nucleic acid (DNA) in the solution was measured by the method of Dische (1930). In this procedure an aliquot of the material was mixed with 3.0 milliliters of diphenylamine reagent (1 gm. diphenylamine, 2 ml. conc. H_2SO_4, and 98 ml. glacial acetic acid) and the volume adjusted to 4.5 milliliters with 5% TCA. The mixture was heated for exactly twenty minutes in a boiling water bath, cooled in running water, and then read against a suitable blank in a Klett-Summerson colorimeter. The amount of DNA present was read from a standard curve (Figure II).

The ribose nucleic acid was determined by the method of Mejbaum (1939). In this procedure the pentose yields a color when treated with orcinol. An aliquot of the solution to be tested was added to 2.4 milliliters of freshly prepared orcinol reagent (100 mg. orcinol and 100 mg. $FeCl_3$ dissolved in 100 ml. conc. HCl) and diluted to five milliliters with 5% TCA. The mixture was heated for exactly twenty minutes, quickly cooled, and read against a blank in the Klett colorimeter.

It was necessary to use the following equation in calculating the concentration of ribose nucleic acid, since desoxyribose nucleic acid

also gave a color with the orcinol reagent.

$$\gamma \text{ RNA·P} = \frac{r-a\ (1.58)}{10.4}$$

Where r is the observed colorimeter reading
a is the γ DNA·P know to be present
1.58 the standard colorimeter reading per γ DNA·P
10.4 the standard reading per γ RNA·P

The standard curves reproduced in FIGURE II were obtained with commercial preparations of the nucleic acids. Approximately 100 mg. of each was separately dissolved in 100 milliliters of 5% TCA by heating. The concentration of the nucleic acids was determined by measuring the phosphorus present (Summer 1944), since the material was too hydroscopic to weigh out accurately. The nucleic acid preparations were checked for inorganic phosphate by suspending 0.5 gm. of each in ten milliliters of water. The suspension was filtered and the filtrate checked for the presence of phosphates. No detectable amount was found. At the concentration used in preparing the standard curve, the ribose nucleic acid gave negligible color with the diphenylamine reagent, indicating that it was relatively free of desoxyribose nucleic acid. No other tests on the purity of the nucleic acid preparations were made.

Hormone Assays.

In the experiments in which the pituitary fractions were to be assayed for hormones, the tissue was not incubated, but was fractionated as soon as the glands were removed from the animals. The pituitaries of thirty to seventy-five adult rats were pooled (three hundred to eight hundred milligrams tissue) and approximately one milliliter of 0.25 M

sucrose was added for every one hundred milligrams of tissue. The material was homogenized, a portion removed for the whole homogenate assay, and the remainder fractionated.

Since the sedimentation rate of the particles is a function of the path length the particles must travel, the large volume of homogenate in these experiments was divided among two or four centrifuge tubes. The portions were fractionated simultaneously in the manner described above. The medium fraction, which consisted entirely of soluble proteins, was presumably recovered in the supernate fraction in these experiments. As soon as the fractions were obtained, they were suspended in 0.25 M sucrose and stored in the refrigerator.

A small portion of each fraction was removed for nitrogen analysis. The remaining portion was assayed for the gonadotropins and growth hormone on hypophysectomized rats. The fractions were injected subcutaneously into groups of the assay animals usually at two or more dose levels, once a day for four days. Control animals received injections of the sucrose solution. All of the animals were sacrificed on the fifth day.

The increase in ovarian weight over the controls was taken as a measure of the gonadotropin activity. In addition, the ovaries were fixed in Bouin's fluid, imbedded in paraffin and sectioned. The serial sections were stained with hematoxylin and eosin and examined for corpora lutea, and antrum follicles. In one experiment male hypophysectomized rats were used for the assay. With these animals the increase in the weight of the seminal vesicles and testes over the controls served

as indication of the gonadotropins. The increase in seminal vesicle weight is believed to be a specific measure of the luteinizing hormone (Fevold 1939).

The method used for the assay of the growth hormone was that of Evans et al. (1943), and consisted essentially of measuring the increase in width of the epiphyseal cartilage of the tibia. The procedure was standardized by Greenspan et al. (1949).

At the time of autopsy the left tibia was removed, split at the proximal end in a sagittal plane, and fixed in 10% neutral formalin (10 ml. of commercial 37% formaldehyde diluted to 100 ml.). One to five days later the bone halves were washed for several hours in four changes of distilled water, and then immersed for one or two hours in acetone. After the acetone had been removed by washing in several changes of distilled water, the tibia halves were immersed in a 2% $AgNO_3$ solution for one and a half minutes. The excess silver nitrate was removed by passing the bones through water. They were then exposed to a strong light (a substage lamp without a filter gave excellent results) until the calcified parts appeared dark brown. The sections were transferred to a 10% sodium thiosulphate solution for one-half minute, and then washed thoroughly in running tap water or several changes of distilled water. After washing, the tibias were stored in 80% ethanol in the dark.

The width of the uncalcified portion of the epiphyseal cartilage was measured under the low power of a microscope with a micrometer eyepiece, calibrated in microns with a stage micrometer. Not less than eight readings at regular intervals were taken across the cartilage, and the

results averaged.

The effect of other hormones on the epiphyseal plate has been studied by Marx et al. (1944). These workers found that only growth hormone could produce an increase of over 20% in the width of the epiphyseal cartilage. Most of the hormones studied produced small increases, but adrenocorticotropin hormone appeared to have an antagonistic effect on the cartilage. In the presence of some hormones the effect of the growth hormone was augmented, and the synergistic action was particularly pronounced in the presence of thyroxin (Becks et al. 1946).

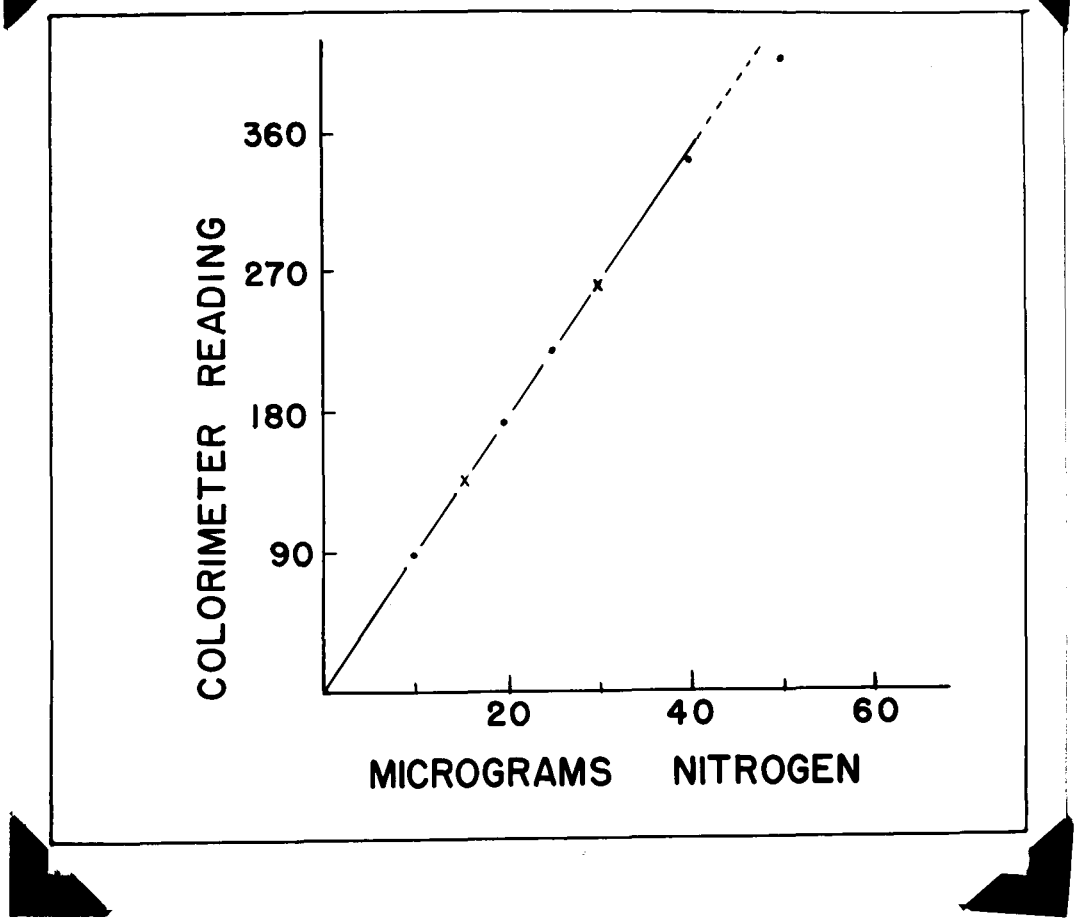


Figure I

STANDARD CURVE FOR NITROGEN DETERMINATION

• Ammonium sulfate standard

x Glycine standard

All of the points are the mean of triplicate determinations.

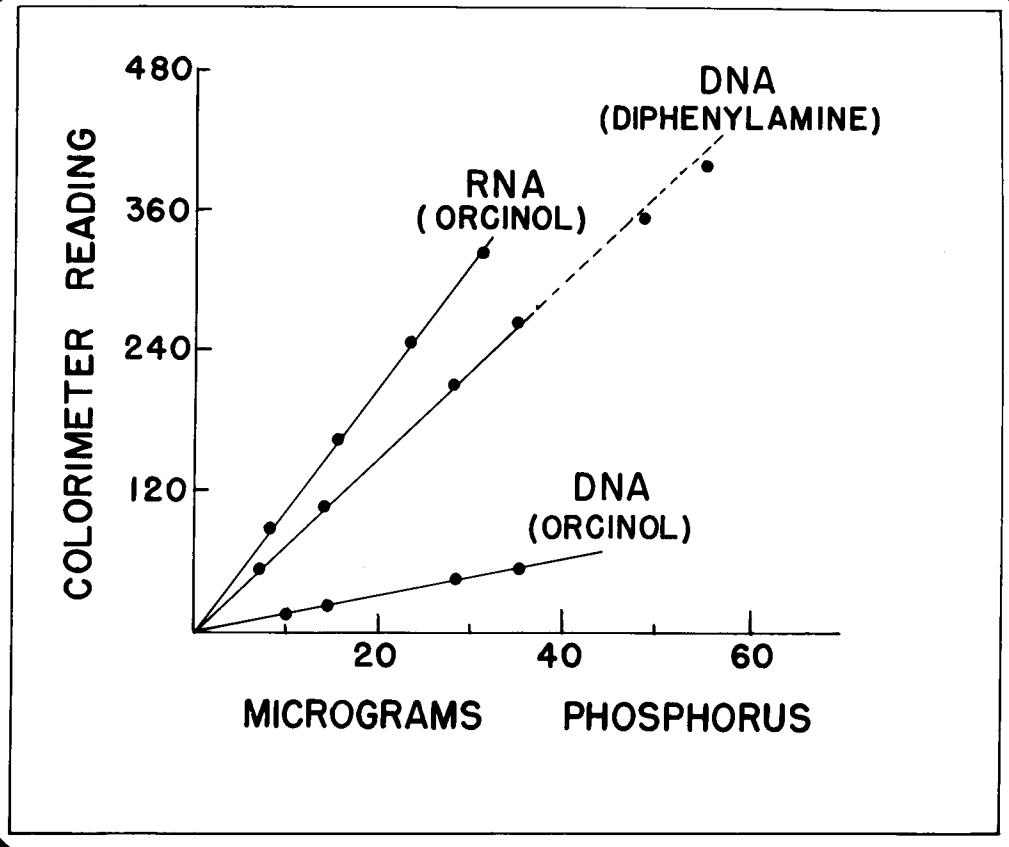


Figure II

STANDARD CURVES FOR NUCLEIC ACID DETERMINATIONS

All of the points are the mean of triplicate determinations.

CHAPTER III

RESULTS AND DISCUSSION

Characterization of the Pituitary Fractions.

The experiments described in this section were performed to determine if the particulate cellular components of pituitary tissue had similar sedimentation characteristics to those reported for liver. It was mentioned previously that in liver the cellular desoxyribose nucleic acid is associated with the nuclei, the ribose nucleic acid with the small granules, and the succinoxidase with the mitochondria (large granules). The distribution of these substances in the pituitary fractions is given in Tables IV and VII.

The bulk of the desoxyribose nucleic acid was recovered in the nuclear fraction; the concentration of this material was over four times as great in this fraction as in the whole homogenate. Microscopic examination of this fraction showed it to consist of nuclei, a few whole cells, and free mitochondria. Contamination of this fraction with mitochondria is also shown by the presence of about 6 per cent of the total succinoxidase activity. However, part of this activity was probably due to the unbroken cells present in this fraction.

The large granule fraction of pituitary tissue consistently contained around 90 per cent of the total succinoxidase activity, and the specific activity of this enzyme was higher in this fraction than in the whole homogenate. Microscopic examination showed this fraction to con-

TABLE IV

DISTRIBUTION OF SUCCINOXIDASE AND NUCLEIC ACIDS IN PITUITARY FRACTIONS

Fraction	Desoxyribose Nucleic Acid		Ribose Nucleic Acid		Succinoxidase	
	DNA·P mg. N	Per-* cent	RNA·P mg. N	Per-* cent	$Q_{O_2}(N)$	Per-* cent
Whole Homogenate	28		18		184 (96-382	
Nuclei	133	96	-	-	46 (0-130)	6 (0-13)
Large Granules	6	3	10	6	620 (310-1200)	89 (86-93)
Small Granules	-	-	66	52	9 (0-28)	-
Supernate	-	-	29	32		
Medium	-	-	-	-	-	-

The nucleic acid data was obtained from one experiment. The succinoxidase values are the average of three separate experiments, and the range is shown by the values in parentheses. In each experiment two or three determinations were made on each fraction.

*These values were calculated on the basis of the material present in the whole homogenate.

tain slightly elongated granules that stained readily with Janus Green B, which is reported to be characteristic of mitochondria (Hogeboom et al. 1948). There also appeared to be a few nuclei in this fraction, which was further verified by the presence of desoxyribose nucleic acid, and numerous spherical granules slightly smaller than the mitochondria, and which were tentatively identified as alpha granules.

The small granule fraction of pituitary tissue contained approximately half of the total ribose nucleic acid of the tissue. In this respect, this fraction is similar to the microsomes obtained from other tissues. The microsomes are submicroscopic, and from their sedimentation rates, they appear to be approximately one hundred to three hundred millimicrons in diameter. Electron microscope studies of liver tissue have revealed that the cytoplasm contains numerous granules, similar in size to the microsomes (Hogeboom and Schneider 1951). It has been suggested (Hogeboom 1951) that the small granule fraction obtained from liver is composed of the submicroscopic particles. This observation presumably can be extended to pituitary tissue.

The supernate fraction, which represents the soluble proteins of the tissue, contained approximately 40 per cent of the total nitrogen when the pituitaries were not incubated before fractionation (Table V). After incubation, only 28 per cent of the protein nitrogen was recovered in this fraction (Table VI). The remaining 12 per cent of the material can be accounted for in the medium fraction, which probably represents the diffusible portion of the soluble proteins. A comparison of the medium fractions of liver and of pituitary tissue shows that nearly twice

TABLE V

FRACTIONATION OF PITUITARY TISSUES BEFORE INCUBATION

Fraction	Conditions	Per Cent Protein* Nitrogen
Nuclei	600Xg - 10 min.	20.4 ±1.0
Large granules	8500xg - 20 min.	19.3 ±0.9
Small granules	24000xg - 3 hours	21.7 ±0.8
Supernate		36.0 ±0.4

*The average of four experiments ± the standard error of the mean.

TABLE VI

DISTRIBUTION OF PROTEIN NITROGEN IN TISSUE FRACTIONS AFTER INCUBATION

Fraction	Conditions[1]	Per Cent Protein Nitrogen[2]	
		Pituitary	Liver
Medium		11.5 ± 0.5	21.5 ± 0.8
Nuclei	600xg - 10 min.	16.8 ±0.8	17.5 ±1.1
Large Granules	8500xg - 20 min.	21.7 ± 0.6	19.1 ±0.5
Small Granules	24000xg - 3 hours	19.0 ± 0.4	19.3 ±1.0
Supernate		28.1 ±0.4	22.8 ±0.5

1). The centrifugal forces given were calculated for the tip of the tube.

2). The average of seven experiments ± the standard error of the mean.

as much protein is lost by the liver cells during incubation. However, the total amount of soluble protein (medium plus supernate) is approximately 40 per cent in each tissue.

The presence of between 20 and 30 per cent of the tissue ribose nucleic acid is reported to be characteristic of the supernate fraction obtained from liver (Hogeboom and Schneider 1951) and is generally attributed to unsedimented microsomes. In this work the supernate fraction in one experiment was subjected to a centrifugal force sufficient to sediment particles larger than fifty millimicrons in diameter. This treatment sedimented approximately 4 per cent of the protein nitrogen, and 30 per cent of the ribose nucleic acid in the supernate fraction (Table VII).

The remaining nucleic acid in the supernatant material was apparently associated with soluble protein. However, this may have been an artifact, since fragmentation of the microsomes could have occurred during homogenization.

From the data discussed in this section, it is evident that a separation of the particulate cellular components of the pituitary can be obtained by differential centrifugation of the tissue homogenate. The nuclei, mitochondria, and microsomes were largely recovered in separate fractions. The distribution of the other particulate elements which can be demonstrated histologically remains in question, with the possible exception of the alpha granules, which may have been recovered in the large granule fraction.

TABLE VII

DISTRIBUTION OF NUCLEIC ACIDS IN PITUITARY FRACTIONS

Fraction	Per Cent* Nitrogen	DNA·P mg. N	Per Cent* DNA	RNA·P mg. N	Per Cent* RNA
Whole Homogenate	-	29.0	-	23.5	-
Nuclei	23.5	108.0	92	-	-
Large Granules	18.0	4	2	11	7
Small Granules-1	19.6	-	-	58	46
Small Granules-2	2.2	-	-	118	11
Supernate	26.8	-	-	15	23

*These values were calculated on the basis of the material in the whole homogenate.
The granule fractions, with the exception of the small granules-2 fraction, were sedimented at forces given in Table V. The small granules-2 fraction was obtained at 110,000xg for thirty minutes.
The tissue was not incubated before fractionation.

Labeling Studies.

Pituitary. The supernate fraction of pituitary tissue contained the largest amount of the labeled methionine and also had the highest specific activity (Table VIII). The rates of incorporation of S^{35} methionine were practically linear with time up to two hours (Figure III), and any deviations from linearity were not significant.

The high rate of incorporation of the label into the supernate fraction of pituitary appears to be characteristic of this tissue, since this was not the case in liver slices (Figure IV). The labeled proteins in the supernate fraction were probably not due to contamination with microsomes, since the distribution of the label was not significantly changed by separating the small granule fraction at 110,000xg for thirty minutes (Table IX).

Attempts to subfractionate the large granule fraction were unsuccessful as is indicated by the data in Table X. The succinoxidase activity and the label were almost equally distributed between the two subfractions.

Liver. The distribution of the radioactivity in the liver fractions was essentially in agreement with reports on the in vivo incorporation of labeled amino acids into fractions of liver tissue separated by differential centrifugation (Hulten 1950; Keller 1951).

A comparison of the rates of label incorporation into the fractions of liver and pituitary shows some striking differences between these two tissues (Figures III and IV), the most pronounced of which were differences in the rates of methionine uptake into the small granule and

TABLE VIII

DISTRIBUTION OF THE S^{35} METHIONINE IN THE PITUITARY FRACTIONS

Fraction	S^{35} Per Cent[1] Methionine	Specific Activity[2] After Incubating	
		1 Hour[3]	2 Hours[4]
Nuclei	11.68 ±0.90	2.17 ±0.19	3.89 ±0.50
Large Granules	19.41 ±0.60	2.80 ±0.40	5.89 ±0.61
Small Granules	22.69 ±0.59	3.76 ±0.23	6.89 ±0.90
Supernate	39.44 ±0.83	4.56 ±0.42	8.05 ±1.01
Medium	6.60 ±1.27	0.58 ±0.42	1.79 ±1.00

1). The average after both one and two hours incubation ± the standard error of the mean.
2). Micromoles labeled methionine per gram protein nitrogen.
3). The average of three experiments ± standard error.
4). The average of four experiments ± standard error.

TABLE IX

SEDIMENTATION OF THE SMALL GRANULES AT 110,000xg

Fraction	Per Cent Label	Specific Activity[1]
Nuclei	13.3	2.98
Large Granules	17.2	6.12
Small Granules[2]	21.1	6.68
Supernate	39.6	7.12
Medium	8.8	3.49

1). Methionine S^{35} per gram protein nitrogen incorporated after two hours.
2). This fraction was sedimented at 110,000xg for thirty minutes.

TABLE X

SUBFRACTIONATION OF THE LARGE GRANULES

Fraction	Conditions	Per Cent Nitrogen[1]	Per Cent Label[1]	Per Cent Succinoxidase[2]
Large Granules-1	4000xg - 10 min.	11.5 ± 0.7	10.1 ± 0.5	44.0 (27-67)
Large Granules-2	8500xg - 20 min.	10.2 ± 0.5	9.8 ± 0.6	44.1 (48-60)

1). Average of five experiments ± standard error of the mean.

2). Average of three experiments and the range is indicated in parentheses.

All of the values in this table are percentages of the total material present in the tissue.

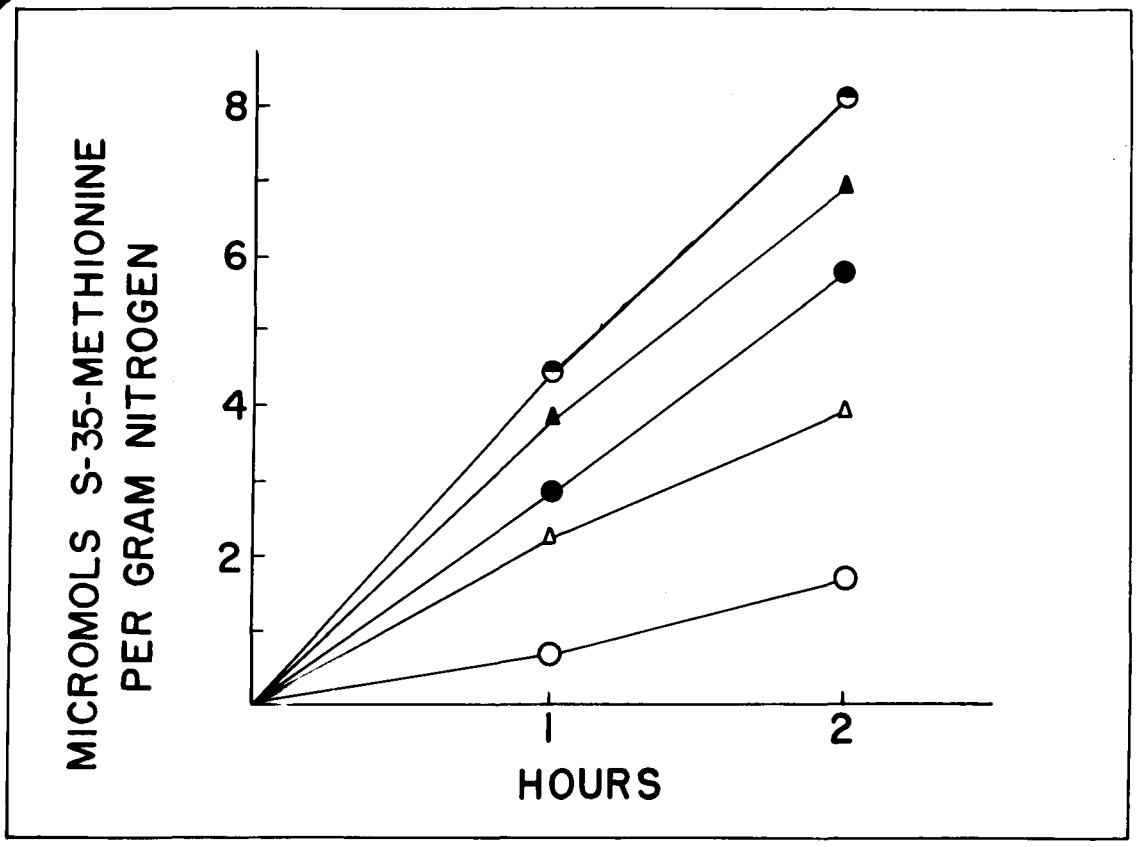


Figure III

PITUITARY PROTEIN LABELING RATE

Location of the label in sub-cellular fractions of pituitary tissue after incorporation by the whole tissue. Δ Nuclei, ● Large granules, ▲ Small granules ◓ Supernate, ○ Medium.

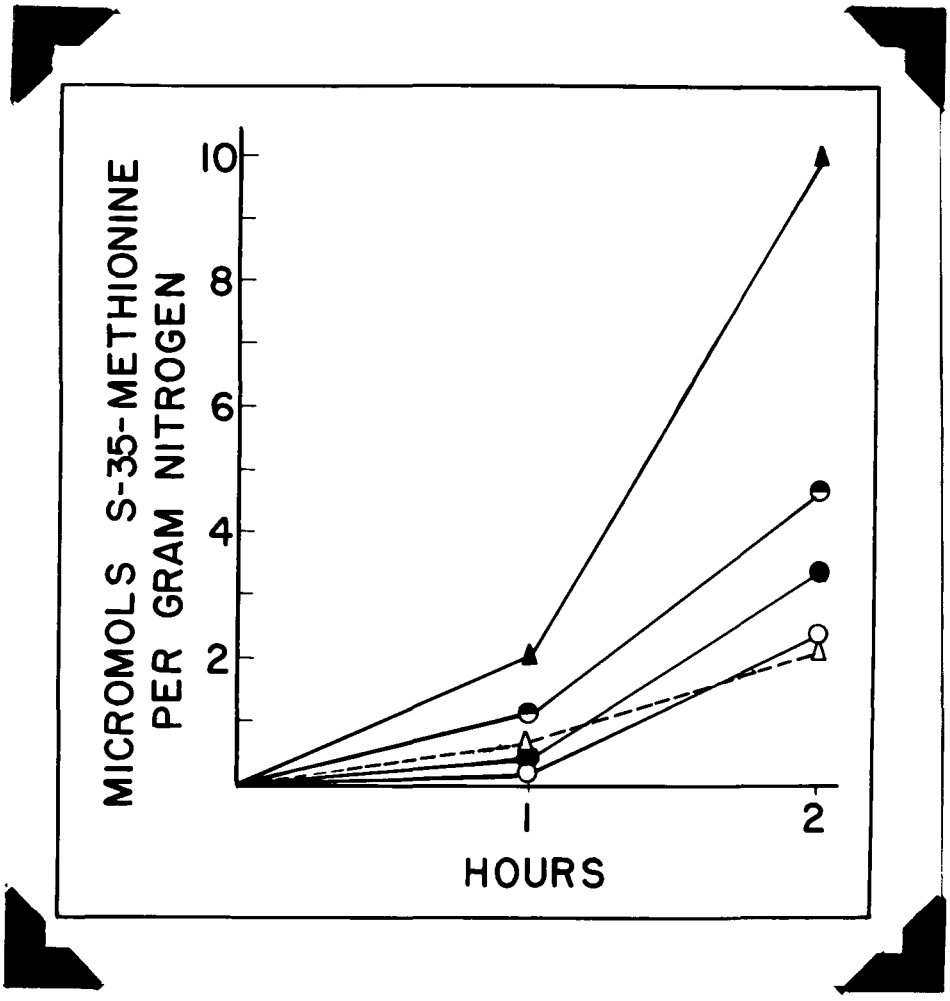


Figure IV

LIVER LABELING RATE

Location of the label in sub-cellular fractions of liver after incorporation into proteins by tissue slices. △ Nuclei, ● Large granules, ▲ Small granules, ◓ Supernate, ○ Medium.

supernate fractions, and the increased rate of protein labeling of the liver fractions during incubation.

After the first hour of incubation, the highest rate of label incorporation occurred in the small granule fraction of liver slices, and this fraction also contained approximately half of the total label incorporated (Table XI). During the second hour of incubation, the small granules still had the highest rate of incorporation, but the per cent of the total radioactivity in this fraction dropped about 7 per cent. This is reflected in the relative change in rates of methionine incorporation into the fraction of liver tissue during incubation.

The measured rates of label uptake into the liver fractions after one and two hours of incubation are shown in Figure IV. From this data it would appear that the rate of protein labeling increased in all of the fractions with time. However, the one and two hour measurements were made with tissue slices from different animals, and there was considerable variation in the total amount of methionine incorporated by the livers of different animals. The liver tissue of two animals used in the two hour incubation experiments were extremely active in the incorporation of the label, and this is shown by the rather large standard error of the mean of the two hour specific activity measurements (Table XI).

While Figure IV does not give the absolute change in the labeling rates of the fractions during incubation, it does show that the relative change in the rates did not occur to the same extent in all of the fractions. The greatest relative change in the rate during incubation

TABLE XI

DISTRIBUTION OF THE S^{35} METHIONINE IN THE LIVER FRACTIONS

Fraction	Per Cent Label After Incubating		Specific Activity* After Incubating	
	1 Hour	2 Hours	1 Hour	2 Hours
Nuclei	8 ±0.4	10 ± 2	0.64 ±0.08	2.2 ± 0.4
Large Granules	10 ±1	14 ±1	0.48 ± 0.04	3.4 ±0.5
Small Granules	45 ±2	38 ± 2	2.01 ± 0.14	10.0 ± 2.1
Supernate	28 ±2	24 ±1	1.18 ±0.10	5.1 ± 1.1
Medium	9 ±1	14 ±1	0.43 ± 0.09	2.7 ±0.5

*Micromoles labeled methionine per gram protein nitrogen.
The one hour incubation values are the average of three determinations and the two hour values the average of four. All are ± the standard error of the mean.

occurred in the large granules and medium fractions, and these two fractions showed a significant increase in the per cent of the total methionine incorporated (Table XI).

The increase in the rate of protein labeling by the liver slices during incubation, and the possible nature of this effect will be considered in a later section of this chapter.

Hormone Assays.

All of the hormone assays were performed on hypophysectomized rats. There was considerable variation in the age and length of time after hypophysectomy of the animals in the different experiments. However, in single experiments none of the animals varied more than fifteen grams in weight, and all had been hypophysectomized the same length of time.

The pituitary tissue in these experiments was obtained from adult female rats, most of which had borne one or more litters. As nearly as could be determined by a superficial examination, these animals were not pregnant or lactating at the time they were used.

Gonadotropins. For the gonadotropin assay listed in the Table XII, immature femal rats, two weeks post-hypophysectomy, were used. These animals ranged in weight from fifty to fifty-eight grams.

Essentially all of the material in each fraction, obtained from 450 milligrams pituitary tissue, was injected into five animals. The rate of injection was graded arithmetically, so that the last animal in each group received five times as much material as the first. Six of the

control animals received injections of 0.25 M sucrose, and the other four were not treated. There was no significant difference between the ovary weights of these two groups of controls. The animals of both control groups were used to obtain the average ovarian weight for controls listed in Table XII.

From the data in Table XII it would appear that all of the fractions, except the nuclear, contained gonadotropic hormones. The greatest increase in ovarian weight occurred in the animals injected with the large granule fraction; however, this fraction produced only about as many follicles as the supernate fraction. The latter fraction was injected at much higher dose levels, but since essentially all of the material in each fraction was used, it would appear that the total amount of follicle stimulating hormone was about the same in the two fractions. The small granule fraction contained considerably less gonadotropin activity than the large granule or supernate fractions. Only the two animals at the nigher dose levels in the small granule fraction group had mature follicles.

Corpora lutea were found in the ovaries of four animals receiving the large granule fraction, while in the whole homogenate only the two animals at the highest dose levels exhibited ovarian corpora lutea. None of the other fractions were able to produce corpora lutea in the animals, and if there was luteinizing hormone in these fractions, the concentration was too low to be detected. From this experiment it would appear that the follicle stimulating hormone was primarily found in two fractions, but the luteinizing hormone was recovered only in the

TABLE XII

GONADOTROPIN ASSAY

Fraction	Dose* (Total Mg. N)	Ovarian Wt. (Mg)	Number of Follicles	Number of Corpora Lutea
Whole Homogenate	0.17 - 0.84	63 (24-101)	20 (7-31)	2 (0-6)
Nuclei and Debris	0.14 - 0.69	14 (11-19)	0 (0-1)	0
Large Granules	0.13 - 0.55	68 (37-97)	18 (2-23)	8 (0-20)
Small Granules	0.10 - 0.50	34 (14-60)	4 (0-15)	0
Supernates	0.23 - 1.14	46 (17-66)	15 (4-24)	0
Controls	-	12 (10-14)	0	0

*The values listed are the highest and lowest levels of injection.

The values in the last three columns are the averages for both ovaries, and the numbers in parentheses indicate the range of the response.

large granule fraction, and was present in this fraction at a higher concentration than in the whole homogenate.

The gonadotropin assays were repeated using male hypophysectomized rats (weight 50-60 grams), two weeks post-hypophysectomy. In this experiment 750 milligrams of pituitary tissue was fractionated. The dosage was adjusted so that each fraction was assayed at approximately the same level of protein nitrogen.

Changes in the weight of the seminal vesicles is believed to be due to the action of testosterone produced by the interstitial cells of the testes under the control of luteinizing hormone. In the absence of testosterone in the material to be assayed, the change in seminal vesicle weight following the injection of the material, is apparently a measure of the luteinizing hormone. Pituitary tissue apparently contains very little testosterone, since injection of pituitary preparation into castrated rats did not produce an increase in the seminal vesicle weight (Fevold 1939).

The results of the gonadotropin assay on male animals are given in Table XIII, and this data is essentially in agreement with that of the previous gonadotropin assay. The largest increase in the weight of the seminal vesicles and testes was observed in the animals receiving the large granule fraction. It is interesting to note that the small granules, which were sedimented at 110,000xg in this experiment, produced as great an increase in the testes weight as the supernate at the same dose level. However, the supernate fraction contained twice as much total protein as the small granule fraction, so the total amount of gonadotropin was

TABLE XIII

GONADOTROPIN ASSAY

Fraction	Dose (Mg. N)	Seminal Vesicles		Testes	
		Average* Weight (Mg.)	Per Cent of Control	Average* Weight (Mg.)	Per Cent of Control
Whole Homogenate	0.19	13 ±1	144	217 ± 26	179
	0.48	14 ±0.2	156	228 ± 21	189
Large Granules	0.16	15 ±1	166	225 ± 25	186
	0.40	17 ±1	189	228 ±17	238
Small Granules	0.22	10 ±1	111	118 ±20	155
	0.56	11 ±2	122	214 ± 6	177
Supernates	0.20	11 ±1	122	181 ± 9	149
	0.48	13 ±1	144	204 ± 7	169
Controls		9 ±0.5		121 ±4	

Four animals were injected at each dose level. The control value represents averages for 10 untreated animals.
*Values are expressed as the mean weights of both organs ± the standard error of the mean.

probably much greater in the soluble fraction.

In two experiments the large granules were subfractionated, and all of the fractions assayed for the gonadotropins. The results of these experiments are listed in Table XIV. The animals used in these experiments weighed between 90-100 grams. In the first experiment listed, the assay animals had been hypophysectomized approximately six weeks before, and in the other experiment, about ten weeks. These animals appeared to be quite refractory to the gonadotropins, but from the relative increases in ovarian weight with the large granules-1 and large granules-2 fractions, it would appear that the gonadotropins were equally distributed between these two fractions.

Growth Hormone. The results of the growth hormone assays are listed in Table XV. In the different experiments the assay animals varied considerably in size and the length of time post-hypophysectomy. There was no assurance that the concentration of growth hormone in the pituitary fraction was the same in the different experiments. Therefore, to correlate this data it was necessary to use the response produced by the whole homogenate as a standard, and to compare the responses obtained with the various fractions directly to the whole homogenate in each experiment.

In Table XV the first column, listed under epiphyseal plate, shows the average width of the left epiphyseal cartilage of the animals at the specified dose levels in each experiment. The increase in width of the epiphyseal plate over the average of the controls in each experiment was calculated, and these values listed in the next column.

TABLE XIV

GONADOTROPIN ASSAYS OF THE LARGE GRANULE SUBFRACTIONS

Fraction	Experiment 24 Dose (Mg. N)	Experiment 24 Ovaries* (Mg.)	Experiment 26 Dose (Mg. N)	Experiment 26 Ovaries* (Mg.)
Whole Homogenate	0.10	26 (12-42)	0.15	11 (6-14)
	0.30	21 (16-24)	0.30	7 (5-10)
Nuclei	0.12	13 (10-14)	0.16	4 (3-5)
	0.36	9 (7-13)	0.31	4 (3-9)
Large Granules-1	0.10	16.4 (14-20)	0.15	9 (7-10)
			0.30	10 (5-15)
Large Granules-2	0.11	18.1 (15-22)	0.10	10 (8-11)
			0.19	11 (8-16)
Small Granules	0.11	15 (9-18)	0.15	5 (3-7)
	0.38	11 (9-13)	0.30	7 (6-8)
Supernates	0.12	13 (11-14)	0.13	10 (5-15)
	0.36	15 (13-16)	0.27	12 (8-17)
Controls		13 (9-14)		5.5 (3-11)

The large granules-1 fraction was sedimented at 4000xg, and the large granules-2 at 85000xg.

*The average weight of both ovaries for the three animals at each dose level, except Experiment 24, where the large granule fractions were injected at one dose level into six animals. The range in ovarian weight is given in parentheses.

TABLE XV

EPIPHYSEAL PLATE MEASUREMENTS

Fraction	Experiment Number	Total Dose (Mg. N)	Epiphyseal Plate Width[2] (Microns)	Epiphyseal Plate Minus[3] Control	Ratio[4] (R)
Whole Homogenate	22 (3)[1]	0.13	133	59	-
	" (3)	0.39	179	105	-
	23 (3)	0.15	183	74	-
	" (3)	0.30	244	135	-
	" (3)	0.60	221	112	-
	25 (3)	0.25	103	36	-
	" (3)	0.61	304	237	-
	27 (4)	0.19	411	203	-
	" (4)	0.48	399	191	-
Nuclei	22 (3)	0.12	93	18	0.31
	" (3)	0.32	107	33	0.31
	23 (4)	0.16	158	49	0.66
	" (4)	0.30	216	107	0.79

TABLE XV (Continued)

Fraction	Experiment Number	Total Dose (Mg. N)	Epiphyseal Plate		Ratio[4] (R)
			Width[2] (Microns)	Minus[3] Control	
Large Granules	22 (4)	0.08	107	33	0.56
	" (4)	0.12	103	29	0.49
	23 (6)	0.15	189	80	1.08
	" (6)	0.30	223	114	0.84
	27 (4)	0.16	348	143	0.70
	" (4)	0.40	349	144	0.75
Small Granules	22 (3)[1]	0.12	112	38	0.64
	" (4)	0.34	202	128	1.21
	23 (3)	0.15	161	52	0.70
	" (3)	0.30	176	67	0.49
	" (3)	0.61	240	131	1.17
	25 (4)	0.23	66	-	0.00
	" (4)	0.56	136	69	0.29
	27 (4)	0.22	287	79	0.39
	" (4)	0.56	282	74	0.39

TABLE XV (Continued)

Fraction	Experiment Number	Total Dose (Mg. N)	Epiphyseal Plate Width[2] (Microns)	Epiphyseal Plate Minus[3] Control	Ratio[4] (R)
Supernate	22 (3)	0.12	151	77	1.31
	" (3)	0.36	181	107	1.01
	23 (3)	0.13	186	77	1.04
	" (3)	0.27	307	198	1.47
	" (3)	0.54	350	241	2.15
	25 (3)	0.22	121	54	1.50
	" (3)	0.60	136	69	0.29
	27 (4)	0.20	384	176	0.88
	" (4)	0.48	407	199	1.04
Controls	22 (10)	-	74		
	23 (13)	-	109		
	25 (7)	-	67		
	27 (8)	-	208		

1). The number of animals.

2). The average of the widths of the left epiphyseal cartilages from the number of animals listed for each dose level.

3). The average width (column 3) minus the average cartilage width of the control animals in the same experiment.

4). See text for definition.

The following relationship was used to obtain a qualitative estimate of the distribution of the growth hormone in the fractions.

$$\frac{(W)_F - (W)_C}{(W)_{W.H.} - (W)_C} = R$$

Where $(W)_F$ = Average width of the epiphyseal cartilage produced by a fraction at a given dose level (mg. protein N).

$(W)_{W.H.}$ = Average width of the epiphyseal cartilage produced by the whole homogenate at the same dose level.

$(W)_C$ = Average width of the epiphyseal cartilage of the control animals in the same experiment.

The values of R for the fractions were determined separately for each experiment, and are listed in the last column of Table XV. These values give an estimate of the growth hormone recovered in each fraction from the whole homogenate.

The supernate was the only fraction that consistently produced an increase in the epiphyseal plates as great as, or greater than, the whole homogenate at the same dose level. In Experiment 27 the small granules were sedimented by centrifuging at 110,000xg for thirty minutes, and this treatment apparently did not significantly alter the growth hormone activity in the small granule and supernate fractions.

The average of all the R values for each fraction in four experiments is listed in Table XVI. These values only indicate the relative concentration of the growth hormone in the fraction. To obtain a qualitative estimate of the toal amount of the growth hormone in the fractions, these values were multiplied by the per cent total nitrogen recovered in the respective fractions, and these figures are also given in Table XVI.

TABLE XVI

DISTRIBUTION OF THE GROWTH HORMONE IN THE PITUITARY FRACTIONS

Fraction	Per Cent Protein N[1]	Growth Hormone	
		Relative Concentration (R)[2]	Total Amount Present[3]
Whole Homogenate		1.00	100
Nuclei	20.4	0.52	10
Large Granules	19.3	0.73	14
Small Granules	21.7	0.59	13
Supernate	38.0	1.19	45

1). Taken from Table V.
2). The average of the values listed in the last column of Table XV. See page 61 of the text for explanation.
3). The values in this column indicate the relative distribution of growth hormone in the fraction obtained from an homogenate that contained 100 arbitrary units.

It would seem that most of the growth hormone was recovered in the supernate fraction.

The log-dose response that was reported to be characteristic of growth hormone assays (Greenspan et al. 1949) was not taken into consideration. However, this factor would not change the qualitative aspects of the data on the distribution of the growth hormone reported here, and would probably emphasize the greater concentration of this hormone in the supernate fraction. It was also impossible to assess the synergistic or antagonistic action of the other hormones present. While the whole homogenate presumably contained all of the pituitary hormones, their influence on the action of the growth hormone may have been different from what it was in the fractions, since the relative concentrations of at least some of the hormones were different in the fractions.

Labeling Studies on Pituitary Tissue from Diethylstilbesterol Treated Animals.

It has been reported (Nelson 1941; Dunning et al. 1947) that chromophobe adenomas of the pituitary could be induced in rats after prolonged treatment with diethylstilbesterol. In these animals there occurred an atrophy of the sex organs and a cessation of growth, indicating a marked change in the physiological function of the pituitary gland. It was, therefore, of interest to determine if the incorporation of labeled methionine by pituitary tissue from similarly treated animals differed significantly from that observed in normal animals.

In these studies male rats, all obtained from the Hormone Assay

Company, were used. Twenty-five milligram pellets of diethylstilbesterol were implanted subcutaneously into the animals once a month for five months. The rest of the animals were not treated and served as controls. Approximately six months after the beginning of the experiment, both the treated and control animals were sacrificed, and the pituitaries removed, labeled, and fractionated in the manner described in Chapter II.

The results of these experiments are listed in Table XVII. The pituitaries of the treated animals were more active in the incorporation of labeled methionine than those of the normal animals, and the distribution of the label was quite different in the two groups. The high rate of protein labeling in the soluble cellular fraction, which is characteristic of normal rat tissue, was not observed in the treated animals. Instead, the specific activity of the labeled proteins was highest in the large and small granule fractions of the abnormal pituitaries.

It has been reported that the labeling enzymes are considerably more active in tumors than in the corresponding normal tissue (Farber et al. 1951). Whether the pituitaries of the diethylstilbesterol treated animals used here actually contained tumors cannot be stated, since microscopic examinations of these glands were not made. The gross appearance of these glands did not differ greatly from those of normal animals. However, some of the larger glands appeared lighter in color and softer in consistency than normal pituitaries.

It is difficult to explain the higher activity of the protein synthesizing enzymes in the pituitaries of the treated animals. The increase in activity probably was not entirely due to proliferation of the

TABLE XVII

PROTEIN LABELING BY THE PITUITARIES OF DIETHYLSTILBESTEROL TREATED ANIMALS

Fraction	Normal Controls[1]		Diethylstilbesterol[2] Treated Animals	
	Per Cent Label	Specific[3] Activity	Per Cent Label	Specific[3] Activity
Nuclei	13.1	2.1	13.0 ±1.3	3.0 ±0.2
Large Granules	17.2	3.8	20.5 ±0.9	7.3 ±0.4
Small Granules	18.7	3.9	27.7 ±1.2	7.1 ±0.4
Supernate	38.6	4.9	34.8 ±1.0	6.1 ±0.5
Medium	12.4	1.8	4.9 ±0.9	2.1 ±0.5

1). One experiment. Average weight of pituitaries was 12.6.
2). Average of three experiments ± standard error of the mean. Average weight of pituitaries of 30 animals was 14.8.
3). Micromoles methionine per gram protein nitrogen after one hour incubation.

tissue, since glands from the treated animals were not appreciably greater in weight than those of the controls. Furthermore, the growth hormone and gonadotropins were probably being produced at a lower rate in these animals since they ceased growing, and the sex organs atrophied. Further experiments are necessary before the nature of this effect can be elucidated.

The Relationship of the Hormones and Labeled Proteins in the Fractions to the Sites in the Intact Cell.

The results of the labeling studies and hormone assays on pituitary tissue are summarized in Table XVIII. It is evident that a partial separation of the protein hormones studied can be obtained by differential centrifugation of the tissue homogenates. A separation of the labeled proteins was also obtained, and approximately 40 per cent of the label was recovered in the soluble fraction at a higher specific activity than in the whole tissue.

The possibility must be considered that the labeled proteins and hormones associated with the isolated granule fractions may not represent the distribution of the material in the intact cells. Nonspecific adsorption of the proteins on the granules could have occurred during the homogenation and fractionation of the tissue. The growth and follicle stimulating activity found in the granule fractions may have been due to adsorption, but it is also possible that these two hormones were originally associated with a particulate cellular component and were merely rendered soluble by homogenization. From the data on these

TABLE XVIII

DISTRIBUTION OF THE LABEL AND HORMONES IN THE PITUITARY FRACTIONS

Fraction	Hormone Activity	Per Cent Label	Rate of Label* Incorporation
Nuclei	Growth +		
	FHS -	12	2.2
	LH -		
Large Granules	Growth +		
	FHS ++	19	2.8
	LH ++++		
Small Granules	Growth +		
	FHS +	22	3.8
	LH -		
Supernate	Growth +++		
	FHS ++	39	4.6
	LH -		
Medium	-	6	1.9

*Micromole of methionine per gram protein nitrogen per hour.

two hormones presented in this work, it is difficult to determine if either of these phenomena occurred.

The presence of the luteinizing hormone in the large granule fraction would be difficult to attribute to non-specific adsorption, since most of it was recovered in this fraction, and a specific adsorption of this hormone on the large granules may be related to the synthesis or storage of this hormone in the intact cell.

The difference in specific activity (micromoles S^{35} methionine per gram nitrogen) of the proteins, observed between the fractions of the normal and diethylstilbesterol treated animals, would indicate that extensive redistribution of the labeled proteins did not occur during fractionation.

While it would be premature to conclude that the protein hormones are actually being labeled in vitro, all of the data presented here is at least consistent with such a view, since both hormones and labeled proteins were found in all of the fractions.

Activation of the Amino Acid Incorporating System of Rat Liver Slices upon Incubation.

During the labeling studies on the liver fractions, it was noted that the rate of methionine incorporation appeared to increase during incubation (Figure IV), and the experiments in this section were preformed to determine the extent of the rate increase and the possible nature of this effect.

In these experiments the tissue was not fractionated, and at

the end of the incubation period the labeling reaction was stopped by adding trichloroacetic acid (final concentration 0.33 M) to the reaction vessel. The washing and counting procedures were the same as described in Chapter II.

The rate of methionine incorporation into liver proteins is shown in Table XIX and Figure V. In each experiment liver slices from a single animal were used to determine the label incorporated after one, two, or three hours of incubation. It is evident that the rate of incorporation was not linear, but increased with time, the values after two hours being approximately three times those after the first hour. This increase in rate appears to be characteristic of liver tissue, since it does not occur with pooled rat pituitaries (Figure V), nor it it apparent in reported data on diaphragm (Borsook et al. 1950c) or bone marrow (Borsook et al. 1950b).

Effect of Incubation Before Addition of the Labeled Methionine. It has been reported that there is a period of approximately twenty minutes between the time of injection, in vivo, of a labeled amino acid and its appearance in serum protein (Green and Anker 1954). Peters (1952) demonstrated a similar type of lag in the appearance of labeled alanine in the albumin produced by rat liver slices in vitro, and postulated an intermediate into which the label must first be incorporated before being used to build the final protein. To determine if the increased uptake observed upon incubation was due to a similar type of phenomenon, slices were incubated for one hour prior to the addition of the labeled methionine. Then the methionine was added, and the incubation continued for one more hour.

TABLE XIX

RATE OF METHIONINE INCORPORATION INTO RAT LIVER PROTEINS

Rat Number	Micromoles Methionine S^{35} per Gram Nitrogen Incorporated After Incubating		
	1 Hour	2 Hours	3 Hours
1	1.8* (2)	5.1 (3)	8.3 (3)
2	1.4 (4)	4.2 (4)	
3	2.6 (3)	7.3 (3)	

*The average of the number of determinations indicated in parentheses.

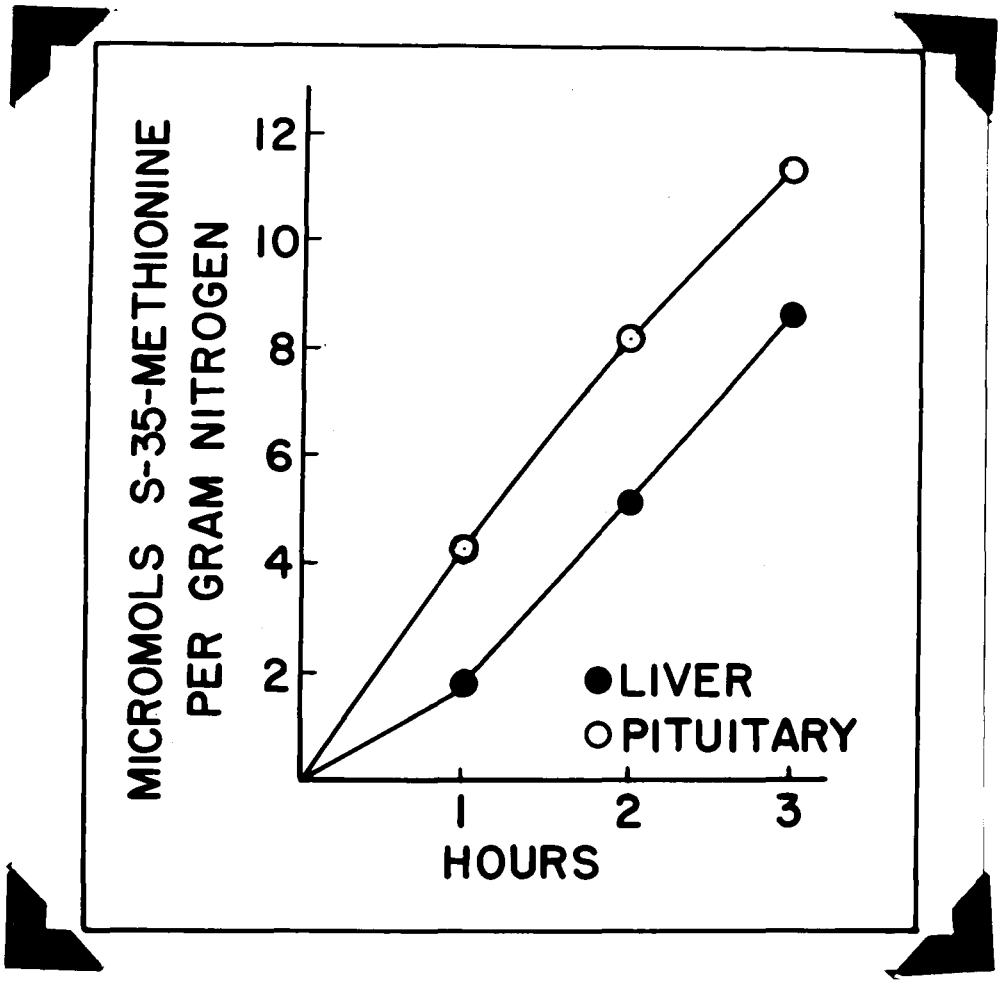


Figure V

THE RATE OF METHIONINE INCORPORATION INTO LIVER AND PITUITARY TISSUE

The pituitary curve was obtained with pooled rat pituitaries, and each point is the average of three determinations. The liver data was taken from Table XIX.

In each experiment the rate of the labeling reaction during the first hour of incubation was determined with liver slices from the same animal as those used in the preincubated flasks.

The results of these experiments are listed in Table XX and Figure VI, and it is quite evident that the presence of the label was not essential to the increased rate of incorporation observed in the second hour. This would seem to preclude the requirement for the synthesis of labeled methionine intermediates as an explanation of the increased rate of protein labeling, and would appear rather to indicate a specific activation of the amino acid incorporating system upon exposure of the slices to Ringer's solution. In the four separate experiments studying the effect of preincubation in Ringer's solution, the average of all values for the first hour (in micromoles methionine incorporated per gram protein nitrogen per hour ±standard error) was 3.4 ±0.2, and for the second hour was 6.3 ±0.4. However, when the ratio of the incorporation during the second hour over the first hour of incubation was calculated in each experiment, and these values averaged, 1.86 ±0.06 was obtained. Thus, although there was some variation in the total amount of label incorporated by liver slices of different animals, the relative increase in the rate during incubation was quite constant for all the animals studied.

Effect of Preincubation in the Absence of Succinate or Oxygen. One experiment was carried out in which the succinate was omitted during the preincubation, and was added with the methionine at the beginning of the labeling reaction. No difference was observed between the flasks

TABLE XX

INCORPORATION OF METHIONINE S^{35} INTO LIVER PROTEINS: EFFECT OF INCUBATION IN RINGER'S SOLUTION

Rat Number	Conditions[1]	Micromoles Methionine-S^{35} Per Gram Nitrogen per Hour	
4	Control	3.1, 3.9, 3.5	(3.5)[2]
	Preincubated	6.3, 6.5, 6.7	(6.5)
5	Control	2.5, 2.6, 2.8	(2.6)
	Preincubated	5.4, 5.6, 5.6	(5.5)
6	Control	3.1, 3.7	(3.4)
	Preincubated	5.8, 6.2, 6.2	(6.0)
	Preincubated Without Succinate	6.3, 6.2, 6.6	(6.3)
7	Control	3.8, 4.6	(4.2)
	Preincubated	6.7, 7.7	(7.2)
	Preincubated Under Nitrogen	1.0, 1.0, 1.0	(1.0)

1). In all cases the labeling reaction was measured for exactly one hour in an atmosphere of O_2-CO_2, and in the presence of succinate. In control samples the labeled methionine was tipped in immediately after the flasks were filled with O_2-CO_2. Preincubations were carried out for one hour before tipping in the methionine. Conditions during preincubation were identical to those during the assay of the incorporating system, except in the last two experiments, where some of the slices were preincubated without succinate or under nitrogen.

2). The average value is shown in parenthesis.

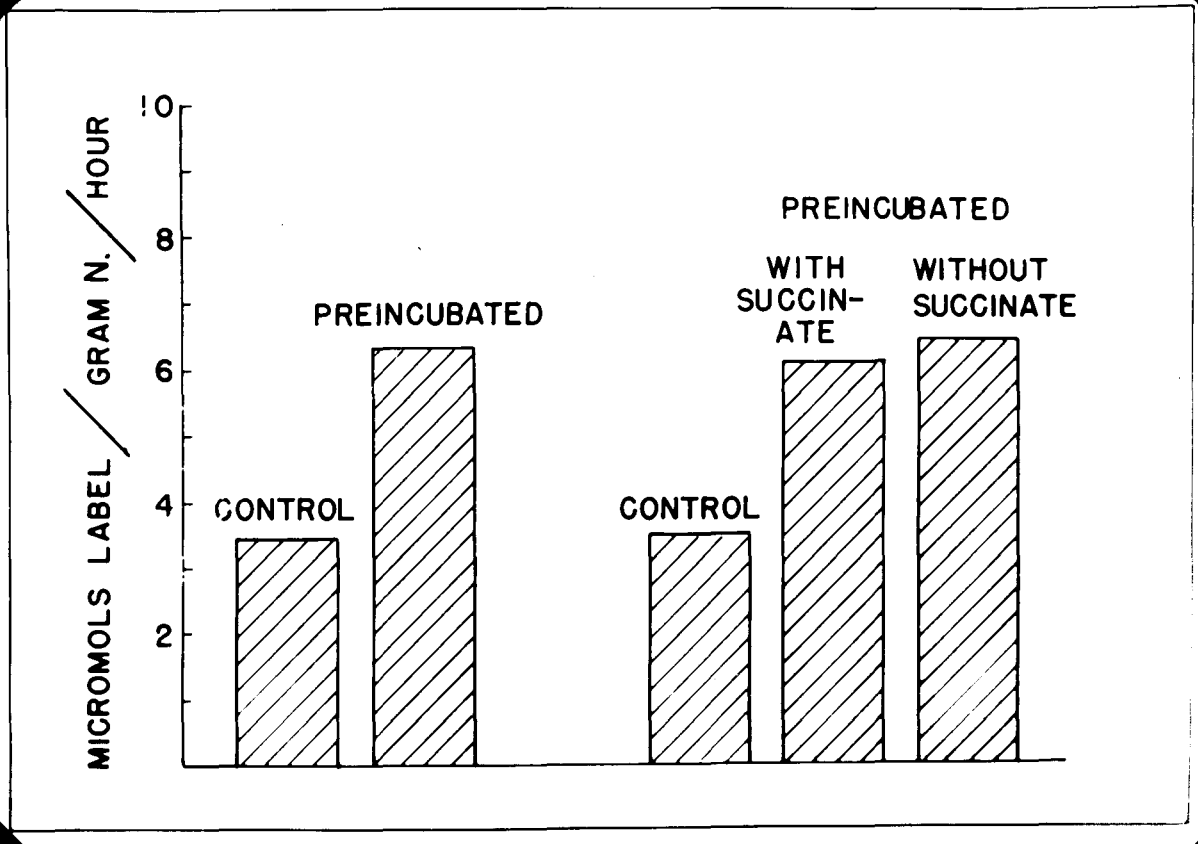


Figure VI

EFFECT OF PREINCUBATION

The two bars on the left represent the average of all the preincubation experiments in Table XX. The three bars on the right is the data from rat number 6 Table XX.

preincubated without and those with succinate, and the results of this experiment are also listed in Table XX and Figure VI.

In another experiment, slices were preincubated for one hour under nitrogen, the flasks being filled with 95% O_2 - 5% CO_2 just before the addition of the methionine. It is evident from the data (Table XX and Figure VII) that preincubation under nitrogen markedly reduces the ability of the slices to incorporate the label. From this data it appears that active oxidation is not only required for amino acid incorporation, but is also necessary to maintain the enzyme system catalyzing the process.

The nitrogen used in this experiment did not contain carbon dioxide, and the pH probably rose in these flasks during the preincubation period. The change in pH in these flasks was not determined, but in other flasks containing similar amounts of tissue, the change in pH after five minutes incubation under nitrogen was found to be a function of the total amount of tissue present. Without tissue the pH was 8.1, with thirty milligrams tissue the pH was 7.8, and with sixty milligrams tissue the pH rose to 7.5. Since the total amount of tissue in the samples measuring the effect of preincubation under nitrogen, varied from approximately thirty to sixty milligrams, it would seem likely that the extensive destruction of the incorporating system was attributable to anaerobiosis during this period, rather than the change in pH.

Effect of Rat Serum As the Incubation Medium. During incubation of the liver slices in Ringer's bicarbonate solution, approximately 20 per cent of the protein leaked into the medium (medium fraction, Table VI). To determine the effect of this loss of protein, two experiments were run

using rat blood serum as the incubation medium. The serum was obtained by pooling blood drawn from the tails of six to eight adult rats. After the blood had clotted, the serum was removed at the centrifuge and used not later than two and one-half hours after it was drawn.

In these experiments the labeling procedure was slightly modified. The methionine was dissolved in distilled water, placed in the side arm of the Warburg flasks and evaporated to dryness at 80°C. When the labeling procedure was to be started, serum was tipped into the sidearm of the flask, shaken in the water bath for approximately one minute, and then transferred back to the main chamber of the vessel. Since the large amount of protein in the serum would interfere in the counting of the samples, the slices were removed from the flasks with forceps, rapidly washed in Ringer's, and dropped into 0.33 M trichloroacetic acid.

Determination of the protein nitrogen of the tissue indicated that the net loss of protein by the tissue during incubation was prevented by using serum as the medium, and Table XXI shows the ratios (times 100) of the protein nitrogen remaining in the tissue after incubation, to the original wet weight of the tissue.

The data in Table XXII shows that the rate of methionine incorporation during the first hour of incubation was similar in slices incubated in serum or Ringer's. However, after one hour in serum, the increase in the labeling rate was significantly less than that observed when Ringer's was used for preincubation (Figure VIII).

The greatly reduced activation observed in serum may be related to the <u>in vivo</u> regulation of serum protein synthesis. It has been shown

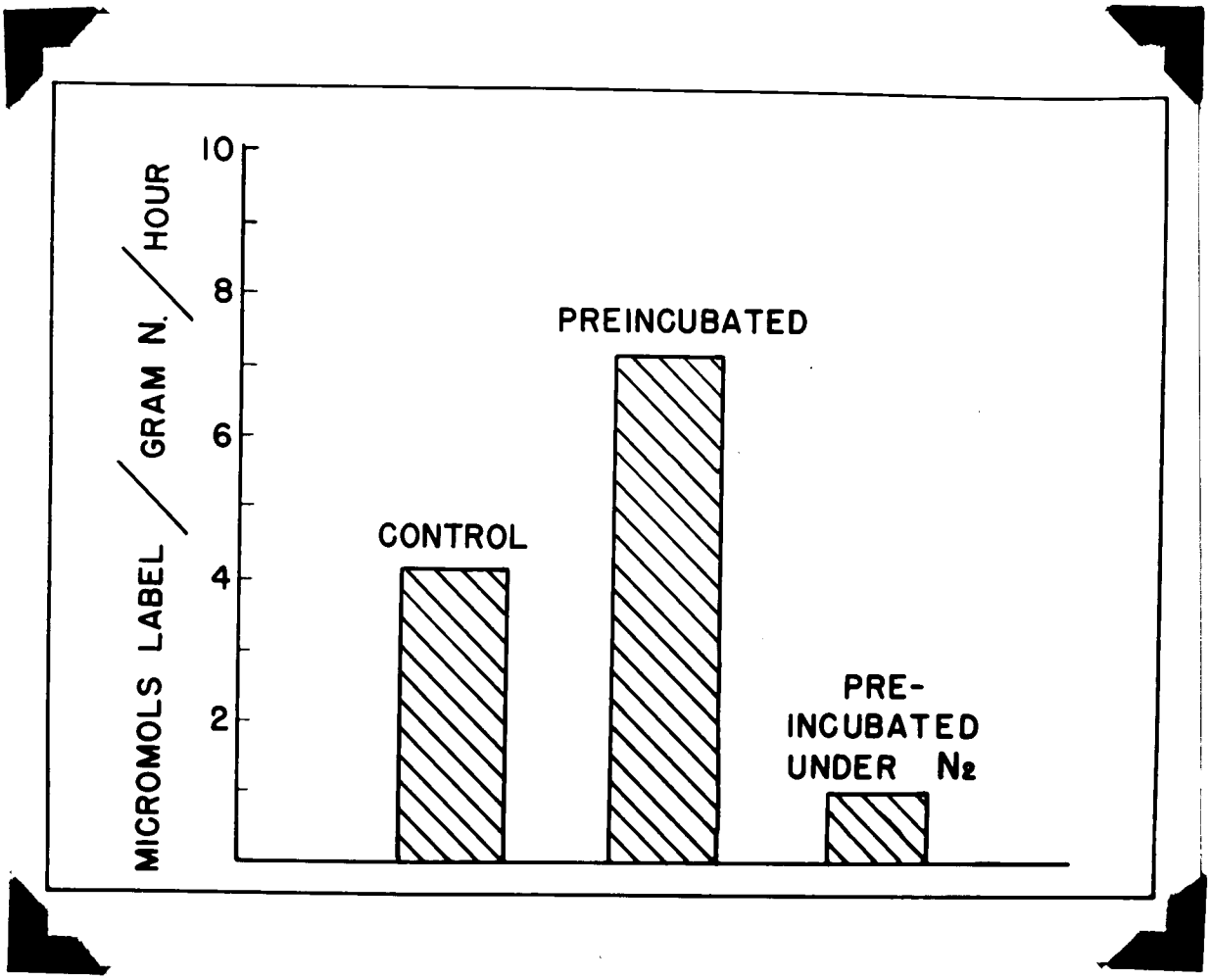


Figure VII

EFFECT OF PREINCUBATION UNDER NITROGEN

The data in this figure is taken from Table XX, animal number 7.

TABLE XXI

EFFECT OF INCUBATION ON NITROGEN CONTENT OF LIVER

Conditions	Per Cent Protein Nitrogen[2]
Fresh Liver (16)[1]	3.1 ± 0.03
After Two Hours Incubation in Ringer's (6)	2.6 ± 0.03
After Two Hours Incubation in Serum (12)	3.0 ± 0.05

1). Number of samples indicated in parentheses.
2). Calculated as milligrams N per 100 Milligrams (wet weight) fresh tissue ± standard error of the mean.

that the liver is quite active in the synthesis of serum proteins (Tarver and Reinhardt 1947), and the rate of synthesis of these proteins is presumably regulated by the level of protein in the serum bathing the liver cells. In the experiments where the incubation medium was Ringer's solution, the liver cells were removed from their normal environment, and placed in a protein free solution. These conditions might be expected to offer a maximal stimulation to protein synthesis by this tissue, reflected in the increased rate of amino acid incorporation.

It is, of course, also possible that the difference in the change in labeling rate observed between the two media may be due to a deleterious effect of Ringer's solution on the cell membrane. If the permeability of the cell membrane increased during the incubation in the Ringer's solution, the rate limiting step during the first hour of incubation could have been the diffusion of the methionine into the cell. However, if this was the case it would appear that the liver cell membranes are more sensitive in this respect than those of pituitary, since with this tissue, the rate of label uptake remained linear with time.

TABLE XXII

EFFECT OF INCUBATION IN SERUM

Rat Number	Conditions[1]	Micromoles Methionine-S^{35} Per Gram Nitrogen Per Hour[2]
8	Control	4.4 ±0.7
	Preincubated	5.8 ±0.4
	Control in Ringer's	4.7, ±5.0
9	Control	4.6 ±0.5
	Preincubated	5.6 ±0.5
	Control in Ringer's	3.9, ±3.6

1). The experiments were similar to those described in Table XIX except that rat serum was used as the medium. For comparison, two flasks of liver slices in Ringer's were run on each animal.

2). All of the values, except the determinations in Ringer's, represent the mean of triplicate determinations ± the standard error of the mean.

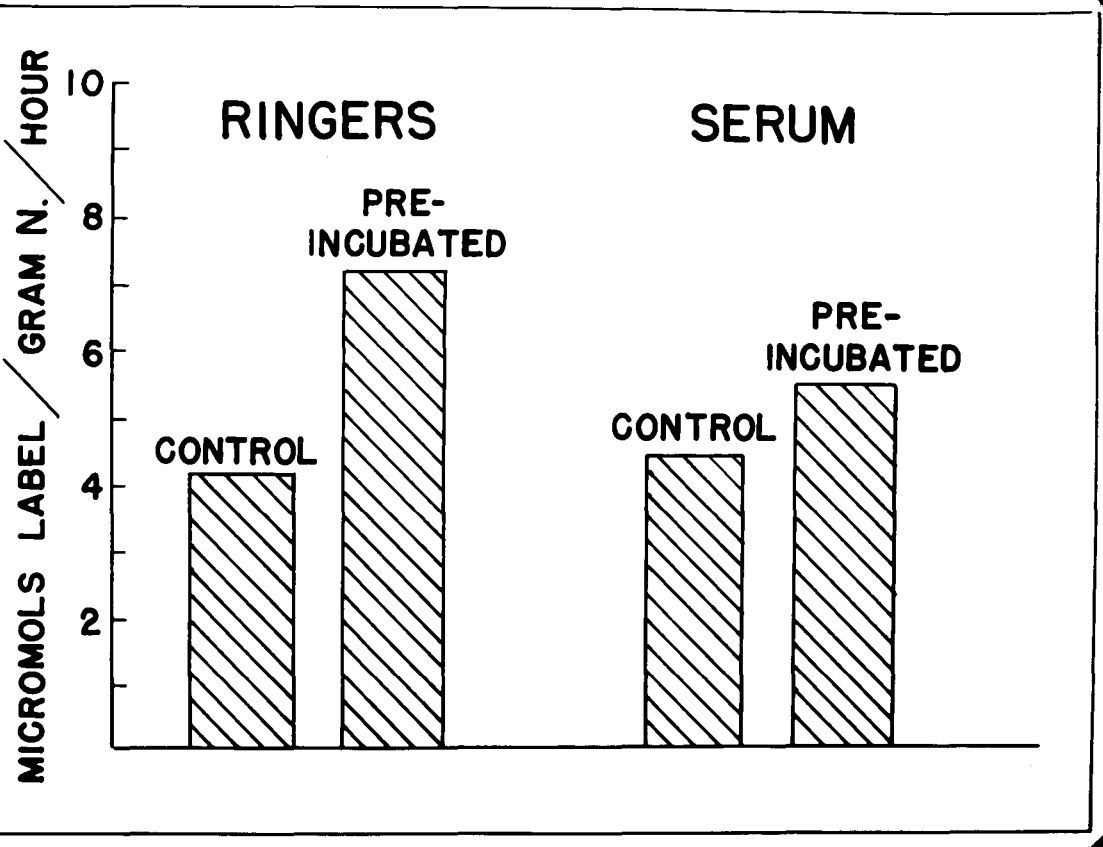


Figure VIII

EFFECT OF PREINCUBATION IN RAT SERUM

The control values represent the label incorporated during the first hour of incubation. The preincubated, the label incorporated during the second hour after one hour of preincubation. For comparison the values from animal number 7, Table XX, obtained in Ringer's are reproduced in this figure.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Rat pituitary was fractionated by differential centrifugation of the tissue homogenate into four fractions; a nuclear fraction, a small granule fraction, a large granule fraction, and a soluble fraction.

The nuclear fraction contained essentially all of the nuclei and desoxypentose nucleic acid of the tissue. The large granule fraction consisted of mitochondria and smaller granules which were tentatively identified as alpha granules; approximately 90 per cent of the tissue succinoxidase activity was recovered in this fraction. The small granule fraction consisted of submicroscopic particles, which had a high ribose nucleic acid content, previously reported to be characteristic of these particles in other tissue.

All of the fractions were assayed for luteinizing, follicle stimulating, and growth hormones. The luteinizing hormone was almost completely recovered in the large granule fraction. Follicle stimulating activity was found in the large granule, small granule, and soluble fractions, with the greatest amounts recovered in the large granule and soluble fractions.

From the characteristics of the pituitary fractions, it can be concluded that this tissue can be separated into its cellular components by differential centrifugation of the tissue homogenate. Resolution of the various particles in the large granule fraction was not obtained.

Pituitary tissue, labeled in vitro with methionine-S^{35} was also fractionated by differential centrifugation, and the characteristics of the granule fractions were the same as those obtained from the fresh tissue, except that during incubation, a portion of the intracellular soluble protein diffused into the medium and was recovered as a separate fraction. The rate of label incorporation into the fractions was linear with time up to two hours, and the highest rate of protein labeling was found in the intracellular soluble fraction. The high rate of protein labeling in the soluble fraction appears to be characteristic of normal pituitary tissue.

The rate of protein labeling and the distribution of the radioactivity in the fractions was markedly altered from normal in pituitary tissue obtained from rats treated with diethylstilbesterol. The rate of methionine incorporation increased in all of the fractions, and the greatest turnover rate was observed in the granule fractions. This is in contrast to normal rat pituitary tissue, where the greatest labeling rate was observed in the soluble fraction.

While it can not be concluded that the protein hormones were being labeled in vitro, all of the data presented here is at least consistent with such a view.

Liver tissue, labeled in vitro with methionine-S^{35} was fractionated by differential centrifugation into its cellular components. With this tissue the highest rate of protein labeling was observed in the small granule fraction. This was in agreement with reports on the in vivo

incorporation of labeled amino acids into liver fractions separated by differential centrifugation.

The rate of methionine incorporation into all of the liver fractions increased during incubation. The greatest increase in the relative rates was observed in the large granule and medium fractions.

The increase in the rate of protein labeling by liver slices was independent of the presence of the label, and was attributed to an activation of the amino acid incorporation system.

Liver slices incubated in rat serum showed a much smaller activation of the amino acid incorporating system, and the possible nature of this effect was discussed.

Preincubation of liver slices under nitrogen resulted in a marked inactivation of the amino acid incorporating system. In the presence of oxygen, endogenous respiration was sufficient to maintain the system.

CHAPTER V

BIBLIOGRAPHY

Anfinsen, C. B., Beloff, A., and Solomon, A. K., 1949, "The Incorporation of Radioactive Carbon Dioxide and Acetate into Liver Proteins *in Vitro*", *J. Biol. Chem.*, 179, 1001.

Becks, H., Simpson, M.E., Evans, H., Ray, R. D., Li, C. H., and Asling, C. W., 1946, "Response to Pituitary Growth Hormone and Thyroxin of the Tibias of Hypophysectomized Rats After Long Postoperative Intervals", *Anat. Rec.*, 94, 631.

Behrens, M., 1932, "Untersuchungen an Isolierten Zellund Gewebsbestandteilen. I. Isolierung von Zellkernen des Kalbsherzmuskels", *Ztschr. f. Physiol. Chem.*, 209, 59.

Bensely, R. R., and Hoerr, N., 1934, "The Preparation and Properties of Mitochondria", *Anat. Rec.*, 60, 449.

Borsook, H., 1950, "Protein Turnover and Incorporation of Labeled Amino Acids into Tissue Proteins *in Vivo* and *in Vitro*", *Physiol. Rev.*, 30, 206.

Borsook, H., Deasy, C. L., Haagen-Smit, A. G., Keighley, G., and Lowy, P. H., 1949, "The Incorporation of Labeled Lysine into the Proteins of Guinea Pig Liver Homogenates", *J. Biol. Chem.*, 179, 689.

Borsook, H., Deasy, C. L., Haagen-Smit, A. G., Keighley, G., and Lowy, P. H., 1950a, "The Uptake *in Vitro* of C^{14}-Labeled Glycine, L-Leucine, and L-Lysine by Different Components of Guinea Pig Liver Homogenates", *J. Biol. Chem.*, 184, 529.

Borsook, H., Deasy, C. L., Haagen-Smit, A. G., Keighley, G., and Lowy, P. H., 1950b, "Incorporation *in Vitro* of Labeled Amino Acids into Bone Marrow Cell Proteins", *J. Biol. Chem.*, 186, 297.

Borsook, H., Deasy, C. L., Haagen-Smit, A. G., Keighley, G., and Lowy, P. H., 1950c, "Incorporation *in Vitro* of Labeled Amino Acids into Rat Diaphragm Proteins", *J. Biol. Chem.*, 186, 309.

Borsook, H. Deasy, C. L., Haagen-Smit, A. G., Keighley, G., and Lowy, P. H., 1950d, "Metabolism of C^{14}-Labeled Glycine, L-Histidine, L-Leucine, and L-Lysine", *J. Biol. Chem.*, 187, 839.

Borsook, H., and Dubnoff, J. W., 1950, "The Biological Synthesis of Hippuric Acid *in Vitro*", *J. Biol. Chem.*, 132, 307.

Brunich, R., and Luck, J. M., 1952, "Amino Acid 'Incorporation' into Protein by Desoxypentose Nucleoprotein and Histone", J. Biol. Chem., 197, 869.

Catchpole, H. R., 1948, "Cell Fractionation and Gonadotropin Assays of Anterior Pituitary Glands", Fed. Proc., 7, 19.

Claude, A., 1943a, "Distribution of Nucleic Acids in the Cell and the Morphological Constitution of Cytoplasm", Biol. Symp., 10, 111.

Claude, A., 1943b, "The Constitution of Protoplasm", Science, 97, 451.

Claude, A., and Potter, J. S., 1943, "Isolation of Chromatin Threads from the Resting Nucleus of Luekemia Cells", J. Exper. Med., 77, 345.

Dhungar, S. B., Sreenivasan, A., 1952, "Synthesis of Xanthine Oxidase by Rat Liver Slices in Vitro", J. Biol. Chem., 197, 831.

Dische, Z., 1930, "Uber Einige Charakteristische Farbreaktionen der Thymonukleinsaure und eine Mikromethode zur Bestimmung derselben in Tierischen Organen mit Hilfe dieser Reaktionen", Mikrochemie, 8, 4.

Donaldson, H. H., 1924, The Rat, 2nd. edition, Philadelphia.

Dounce, A. L., 1943, "Enzyme Studies on Isolated Cell Nuclei of Rat Liver", J. Biol. Chem., 147, 685.

Dounce, A. L. Tishkoff, G. H., Barnett, B. R., and Freer, R. M., 1950, "Free Amino Acids and Nuclei Isolated by a Modification of the Behrens' Technique", J. Gen. Physiol. 33, 629.

Dunning, W. F., Curtis, M. R., and Segaloff, A., 1947, "Strain Differences in Response to Diethylstilbesterol and the Induction of Mammary Gland and Bladder Cancer in the Rat", Cancer Research, 7, 511.

Evans, H., and Long, C. N. H., 1921, "On the Association of Continued Cornification of the Vaginal Mucosa with the Presence of Large Vesicles in the Ovary and the Absence of Corpus Formation", Anat. Rec., 21, 62

Evans, H. M., Simpson, M. E., Marx, W., and Kibrich, E., 1943, "Bio-assay of the Pituitary Gland Hormone. Width of the Epiphyseal Cartilage of the Tibia in Hypophysectomized Rats", Endocrin, 32, 13.

Farber, E. Kit, S., and Greenberg, D. M., 1951, "The Uptake of Radioactive Glycine into Tumor Protein", Cancer Research, 11, 490.

Fevold, H. L., 1939, "Extraction and Standardization of Pituitary Follicle Stimulating and Luteinizing Hormones", Endocrin. 24, 435.

Frantz, I. D., Loftfield, R. C., and Miller, E.N., 1947, "Incorporation of C^{14} from Carboxyl-labeled dl-Alanine into Protein of Liver Slices", Science, 106, 544.

Frantz, I. D., Zamecnik, P. C., Reese, J. W., and Stephenson, M. L., 1948, "The Effect of Dinitrophenol on the Incorporation of Alanine Labeled with Radioactive Carbon into Proteins of Slices of Normal and Malignant Rat Liver", J. Biol. Chem., 174, 773.

Freer, R. M., 1950, "Free Amino Acids and Nucleic Acid Content of Cell Nuclei Isolated by a Modification of Behrens' Technique", J. Gen. Physiol., 33, 629.

Green, H., and Anker, H. S., 1954, "Time Required for Serum Protein Synthesis", Fed. Proc., 13, 220.

Greenspan, F. S., Li, C. H., Simpson, M. E., and Evans, H. M., 1949, "Bioassay of the Hypophyseal Growth Hormone: The Tibia Test", Endocrin., 45, 455.

Hawk, P. B., and Bergeim, O., 1937, Practical Physiological Chemistry, 11th edition, Maple Press Co., York, Pennsylvania.

Herlant, M., 1952, "Les Granulations Acidophils de l'Hypophyse et leur Signification Physiologique", Compt. Rend. Soc. Biol. 146, 1280.

Hogeboom, G. H., 1951, "Separation and Properties of Cell Components", Fed. Proc., 10, 640.

Hogeboom, G. H., Schneider, W. C., and Pallade, G. E., 1948, "Cytological Studies of Mammalian Tissue. I. Isolation of Intact Mitochondria from Rat Liver; Some Biochemical Properties of Mitochondria and Submicroscopic Particulate Material", J. Biol. Chem., 172, 619.

Hokin, L. E., 1951a, "Amino Acid Requirements of Amylase Synthesis by Pigeon Pancreas in Vitro", Biochem. J., 48, 320.

Hokin, L. E., 1951b, "Amino Acid Requirements of Amylase Synthesis by Pigeon Pancreas Slices", Biochem. J., 50, 216.

Hokin, L. E., 1952, "The Role of Ribonucleic Acids in Amylase Secretion by Pancreas Slices", Biochem. et Biophys. Acta, 8, 225.

Hulten, T., 1950, "Incorporation in Vivo of N^{15}-labeled Glycine into Liver Fractions of Newly Hatched Chicks", Exptl. Cell Research, 1, 376.

Hurst, V., and Turner, C. W., 1942, "Lactogenic Hormone Content of Anterior Pituitary Glands of Albino Mice as Compared to Other Species", Endocrin., 31, 334.

Johnson M. B., and Bloch, K., 1951, "Enzymatic Synthesis of Glutathione", J. Biol. Chem., 188, 221.

Keller, E. B., 1951, "Turnover of Proteins of Cell Fractions of Adult Rat Liver in Vivo", Fed. Proc., 10, 206.

Kennedy, E. P., and Lehninger, A. L., 1949, "Oxidation of Fatty Acids and Tricarboxylic Acid Cycle Intermediates by Isolated Rat Liver Mitochondria", J. Biol. Chem., 179, 957.

Kit, S. and Greenberg, D. M., 1951, "Energy Yielding Reactions and Amino Acid Uptake into Protein of the Tumor Cell", Cancer Research, 11, 495.

Kit, S., and Greenberg, D. M., 1952, "Incorporation of Isotonic Threonine and Valine into the Protein of Rat Liver Particles", J. Biol. Chem., 194, 377.

Li, C. H., Simpson, M. E., and Evans, H. M., 1942, "Isolation of Adrenocorticotropic Hormone from Sheep Pituitaries", Science, 96, 450.

Li, C. H., Evans, H. M., and Simpson, M. E., 1943, "Adrenocorticotropic Hormone", J. Biol. Chem., 149, 413.

Li, C. H., and Evans, H. M., 1944, "The Isolation of Pituitary Growth Hormone", Science, 99, 183.

Li, C. H., Evans, H. M., and Simpson, M.E., 1945, "Isolation and Properties of the Anterior Hypophyseal Growth Hormone", J. Biol. Chem., 159, 353.

Linderstrøm-Lang, K. U., 1952, "Biological Synthesis of Proteins; Proteins and Enzymes: Lane Medical Lectures", Stanford University Pub. Med. Sci., 6, 93.

Loeb, L. and Bassett, R. B., 1930, "Comparison of Effects of Various Preparations of Anterior Pituitary Gland on Thyroid of Guinea Pig", Proc. Soc. Exptl. Biol. Med., 27, 490.

Loeb, L., and Bassett, R. B., 1929, "Effect of Hormones of the Anterior Pituitary on Thyroid Gland in the Guinea Pig", Proc. Soc. Exptl. Biol. Med., 26, 860.

Lyons, W. R., 1942, "The Direct Mammotropic Action of Lactogenic Hormone", Proc. Soc. Exptl. Biol. Med. 51, 308.

Marx, W., Simpson, M. E., and Evans, H., 1944, "Specificity of the Epiphyseal-Cartilage Test for the Pituitary Growth Hormone", Proc. Soc. Exptl. Biol. Med., 55, 250.

McShan, W. H., and Meyer, R. K., 1949, "Gonadotropic Activity of Granules Isolated from Rat Pituitary Glands", Proc. Soc. Exptl. Biol. Med., 71, 407.

McShan, W. H., and Meyer, R. K., 1953, "Biochemical Properties of Fractions Obtained from Rat Anterior Pituitary Glands by Differential Centrifugation", Endocrin., 52, 215.

Mejbaum, W., 1939, "Uber die Bestimmung Kleiner Pentosemengen, Insbeondere in Derivaten der Adenylsaure", Hoppe-Seyler's Zeitschrift fur Physiol. Chem., 258, 117.

Melchior, J. B. 1946, "Studies in Sulfur Metabolism Using Labeled Cystine and Methionine", Doctorate Thesis, University of California.

Melchior, J. B., and Goldkamp, A. H., 1954, "Quantitative Determination of S^{35}-Labeled Compounds in Complex Mixtures", Anal. Chem., 26, 233.

Melchior, J. B., and Halikis, M. N., 1952, "The Incorporation of Labeled Methionine into Protein by Pituitary Tissue", J. Biol. Chem., 199, 773.

Melchior, J. B., Mellody, M., and Klotz, I. M., 1948, "The Synthesis of Protein by Non-proliferating Escherichia Coli", J. Biol. Chem., 174, 81.

Melchior, J. B., and Tarver, H. 1947a, "Studies in Protein Synthesis in Vitro. I. On the Synthesis of Labeled Cystine (S^{35}) and its Attempted use as a Tool in the Study of Protein Synthesis", Arch. Biochem. 12, 301.

Melchior, J. B., and Tarver, H., 1947b, "Studies on Protein Synthesis in Vitro. II. On the Uptake of Labeled Sulfur by the Proteins of Liver slices Incubated with Labeled Methionine (S^{35})", Arch. Biochem., 12, 309.

Melchior, J. B., and Sliwinski, B. A., 1954, "Proteolytic Inhibitors in Serum. I. Effect of Food Intake and Hypophysectomy", Cancer Research, 14, 677.

Meyer, R. K., and McShan, W. H., 1952, "Gonadotropic Activity of Granule Fractions Obtained from Anterior Pituitary Glands of Castrate Rats", Endocrin., 50, 294.

Miller, L. L., Bale, W. F., Yuile, C. L., Masters, R. E. Tishkoff, G. H., and Wipple, G. H., 1949, "The Use of Radioactive Lysine in Studies of Protein Metabolism", J. Exptl. Med., 90, 297.

Miller, G. L., and Du Vigneaud, V., 1937, "The Cystine Content of Insulin", J. Biol. Chem., 118, 101.

Mirsky, A. E., and Pollister, A. W., 1943, "Fibrous Nucleoproteins of Chromatin", Biol. Symp., 10, 247.

Mirsky, A. E., and Ris, H., 1947a, "Isolated Chromosomes", J. Gen. Physiol., 31, 1.

Mirsky, A. E., and Ris, H., 1947b, "The Chemical Constitution of Isolated Chromosomes", J. Gen. Physiol., 31, 7.

Nelson, W. O., 1941, "The Occurrence of Hypophyseal Tumors in Rats Under Treatment with Diethylstilbesterol" Am. J. Physiol., 133, 398.

Peters, T. 1952, "Evidence of Intermediate Compounds in Serum Albumin Synthesis", J. Biol. Chem., 200, 461.

Peters, T., and Anfinsen, C. B., 1950a, "Production of Radioactive Serum Albumin by Liver Slices", J. Biol. Chem., 182, 171.

Peters, T., and Anfinsen, C. B., 1950b, "Net Production of Serum Albumin by Liver Slices", J. Biol. Chem., 186, 805.

Peterson, E. A., and Greeberg, D. M., 1952, "Characteristics of the Amino Acid Incorporating System of Liver Homogenates", J. Biol. Chem., 194, 395.

Peterson, E. A., Winnick, T., and Greenberg, D. M., 1951, "Stimulation by the Adenylic Acid System of Amino Acid Incorporation into Protein of Liver Granules", J. Am. Chem. Soc., 73, 503.

Potter, V. R., and Elvehjem, C. A., 1936, "A Modified Method for the Study of Tissue Oxidation", J. Biol. Chem., 114, 495.

Ranney, H. M., and London, I. M., 1951, "Antibody Formation in Surviving Tissues", Fed. Proc. 10, 562.

Sayers, G., White, A., and Long, C. N. H., 1943, "Preparation and Properties of Pituitary Adrenotropic Hormone", J. Biol. Chem., 149, 425.

Schneider, W. C., 1945, "Extraction of Desoxypentose Nucleic Acid and Pentose Nucleic Acid", J. Biol. Chem., 161, 293.

Schneider, W. C., 1948, "Intracellular Distribution of Enzymes. III. The Oxidation of Octanoic Acid by Rat Liver Fractions", J. Biol. Chem., 176, 259.

Schneider, W. C., Claude, A., and Hogeboom, G. H., 1948, "The Distribution of Cytochrome C and Succinoxidase Activity in Rat Liver Fractions", J. Biol. Chem., 172, 451.

Schneider, W. C., and Hogeboom, G. H., 1950, "Intracellular Distribution of Enzymes. II. Further Studies on the Distribution of Cytochrome C in Rat Liver Homogenates", J. Biol. Chem., 183, 123.

Schneider, W. C., and Hogeboom, G. H., 1951, "Cytochemical Studies of Mammalian Tissues: The Isolation of Cell Components by Differential Centrifugation", Cancer Research, 11, 1.

Schneider, W. C., Hogeboon, G. H., and Ross, M. H., 1950, "Intracellular Distribution of Enzymes. VII. The Distribution of Nucleic Acids and Adenosinetriphosphate in Normal Mouse Liver and Mouse Hepatoma", J. Nat. Cancer Inst., 10, 977.

Schneider, W. C., and Potter, V. R., 1943, "The Assay of Animal Tissue for Respiratory Enzymes", J. Biol. Chem., 149, 217.

Schwett, R., and Borsook, H., 1953, "Incorporation of Radioactive Glycine into Protein", Fed. Proc., 12, 266.

Selye, H., 1949, Textbook of Endocrinology, 2nd edition, Montreal.

Siekewitz, P., 1952, "Uptake of Radioactive Alanine in Vitro into Proteins of Rat Liver Fractions", J. Biol. Chem, 195, 549.

Simpson, M. V., and Tarver, H., 1950, "Studies on Protein Synthesis in Vitro. III. Further Observations on the Incorporation of Methionine into Liver Protein", Arch. Biochem., 25, 384.

Smelser, G. K., 1944, "Differential Concentration of Hormones in the Central and Peripheral Zones of the Bovine Anterior Pituitary Gland", Endocrin., 34, 39.

Smith, P. E., 1927, "The Experimental Feeding of Fresh Anterior Pituitary Substance to the Hypophysectomized Rat", Am. J. Physiol., 81, 20.

Smith, P. E., 1930, Hypophysectomy and Replacement Therapy in the Rat", Am. J. Anat., 45, 205.

Smith, P. E., and Engel, E. T., 1927, "Experimental Evidence Regarding the Role of the Anterior Pituitary in the Development and Regulation of the Genital System", Am. J. Anat., 40, 159.

Striker, P., and Greuter, T., 1928, "Action du Lobe Anterieur de L'Hypophyse sur la Montee Loiteuse", Compt. Rend. Soc. Biol., 99, 1978.

Summer, J. B., 1944, "A Method for the Colorimetric Determination of Phosphorus", Science, 100, 413.

Tarver, H., 1954, The Proteins, Vol. II., Part B, 1276, edited by H. Neurath, and K. Bailey, New York.

Tarver, H., and Reinhardt, W. O., 1947, "Methionine Labeled with Radioactive Sulfur As an Indicator of Protein Formation in the Hepatectomized Dog", J. Biol. Chem., 167, 395.

Thompson, J. F., and Morrison, G. R., 1951, "Determination of Organic Nitrogen", Anal. Chem., 23, 1153.

Thorn, G. W., Forsham, P. H., Prunty, F. T. G., and Hills, A. G., 1948, "A Test for Adrenocortical Insuffiency", J. Am. Med. Assoc., 137, 1005.

Umbreit, W. W. Burris, R. H., and Stauffer, J. F., 1949, Manometric Techniques and Tissue Metabolism, Chapter XI, 139. Burgess Publishing Co., Minneapolis.

White, A., 1944, Chemistry and Physiology of Hormones, American Association for the Advancement of Science, Washington, D. C.

White, A., Catchpole, H. R., and Long, C. N. H., 1937, "A Crystalline Protein with High Lactogenic Activity", Science, 86, 82.

Winnick, T., 1950a, "Studies on the Mechanism of Protein Synthesis in Embryonic and Tumor Tissues. I. Evidence Relating to the Incorporation of Labeled Amino Acids into Protein Structure in Homogenates", Arch. Biochem., 27, 65.

Winnick, T., 1950b, "Studies on the Mechanism of Protein Synthesisin Embryonic and Tumor Tissues. II. Inactivation of Fetal Rat Liver Homogenate by Dialysis, and Reactivation by the Adenylic Acid System", Arch. Biochem., 28, 338.

Winnick, T., Friedberg, F., and Greenberg, D. M., 1947, "Incorporation of C^{14}-labeled Glycine into Intestinal Tissue and Its Inhibition by Azide", Arch. Biochem, 15, 160.

Winnick, T., Friedberg, F., and Greenberg, D. M., 1948, "The Utilization of Labeled Glycine in the Process of Amino Acid Incorporation by the Protein of Liver Homogenates", J. Biol. Chem., 175, 117.

Winnick, T., Peterson, E. A., and Greenberg, D. M., 1949, "Incorporation of C^{14}-Glycine into Protein and Lipide Fractions of Homogenates", Arch. Biochem., 21, 235.

Zamecnik, P. C., Frantz, I. D. Loftfield, R. B., and Stephenson, M. L. 1948, "Incorporation in Vitro of Radioactive Carbon from Carboxyl Labeled dl-Alanine and Glycine into Proteins of Normal and Malignant Rat Tissue", J. Biol. Chem., 175, 299.

Zittle, C. A., and O'Dell, R. A., 1941, "The Determination of Cystine", J. Biol. Chem., 139, 753.

Zondek, B., and Ascheim, S., 1927, "Das Hormon des Hypophysenvorden-Lappens", Klin. Wochschr., 6, 248.

CHAPTER VI

APPENDIX

Hormone Assays

Experiment 6

The assay animals were female hypophysectomized rats and were used two weeks post-hypophysectomy. Four hundred and ninety four milligrams pituitary tissue was fractionated in the manner described in Chapter II except that the small granule fraction was sedimented at 18,500 x g for 1 hour.

Animal No.	Dose (Mgs. N)	Body Weight (Gms.)	Weight of Organs (Mgs.)				Width of Epiphseal Plate (Microns)
			Ovaries	Uterus	Adrenals	Thyroids	
Whole Homogenate							
22	0.17	50	24	100	12	4.6	303
03	0.34	50	73	102	9	6.6	296
12	0.50	53	66	106	8	5.3	368
02	0.67	54	85	90	13	6.8	-
41	0.84	57	101	71	14	-	326
Nuclei							
35	0.14	49	19	25	8	5.0	242
20	0.28	52	13	20	9	6.0	282
13	0.41	53	19	28	14	4.5	339
40	0.55	54	11	32	12	-	-
50	0.69	53	11	22	10	5.6	306

Experiment 6 (Cont'd)

Animal No.	Dose (Mgs. N)	Body Weight (Grams)	Weight of Organs (Mgs.)				Width of Epiphseal Plate
			Ovaries	Uterus	Adrenals	Thyroids	
Large Granules							
04	0.13	56	37	82	14	4.7	204
21	0.26	53	60	111	11	5.8	311
01	0.39	53	66	62	13	6.2	246
11	0.52	52	97	64	12	7.4	351
54	0.55	51	80	67	10	6.1	306
Small Granules							
15	0.09	56	14	11	8	4.2	201
14	0.17	52	-	23	12	4.5	293
32	0.26	52	34	80	12	5.4	229
33	0.34	57	28	32	12	5.7	216
31	0.43	55	60	108	18	6.2	365
Supernate							
44	0.23	50	17	19	11	4.1	297
53	0.46	52	66	95	11	7.5	270
43	0.68	53	41	105	15	4.9	233
51	0.91	55	50	116	16	6.5	358
30	1.14	55	58	71	10	5.2	306
Controls							
55	-	52	10	12	9	4.0	195
05	-	53	10	23	11	5.1	158
34	-	53	10	16	14	4.2	213

Experiment 6 (Cont'd)

Animal No.	Dose (Mgs. N)	Body Weight (Gms.)	Weight of Organs (Mgs.) Ovaries	Uterus	Adrenals	Thyroids	Width of Epiphseal Plate
25	-	54	13	19	8	4.8	177
10	-	56	15	24	13	4.1	145
Untreated Controls							
	-	40	10	10	6	3.8	79
	-	41	7	12	6	3.9	110
	-	44	8	-	7	3.7	89

Experiment 22

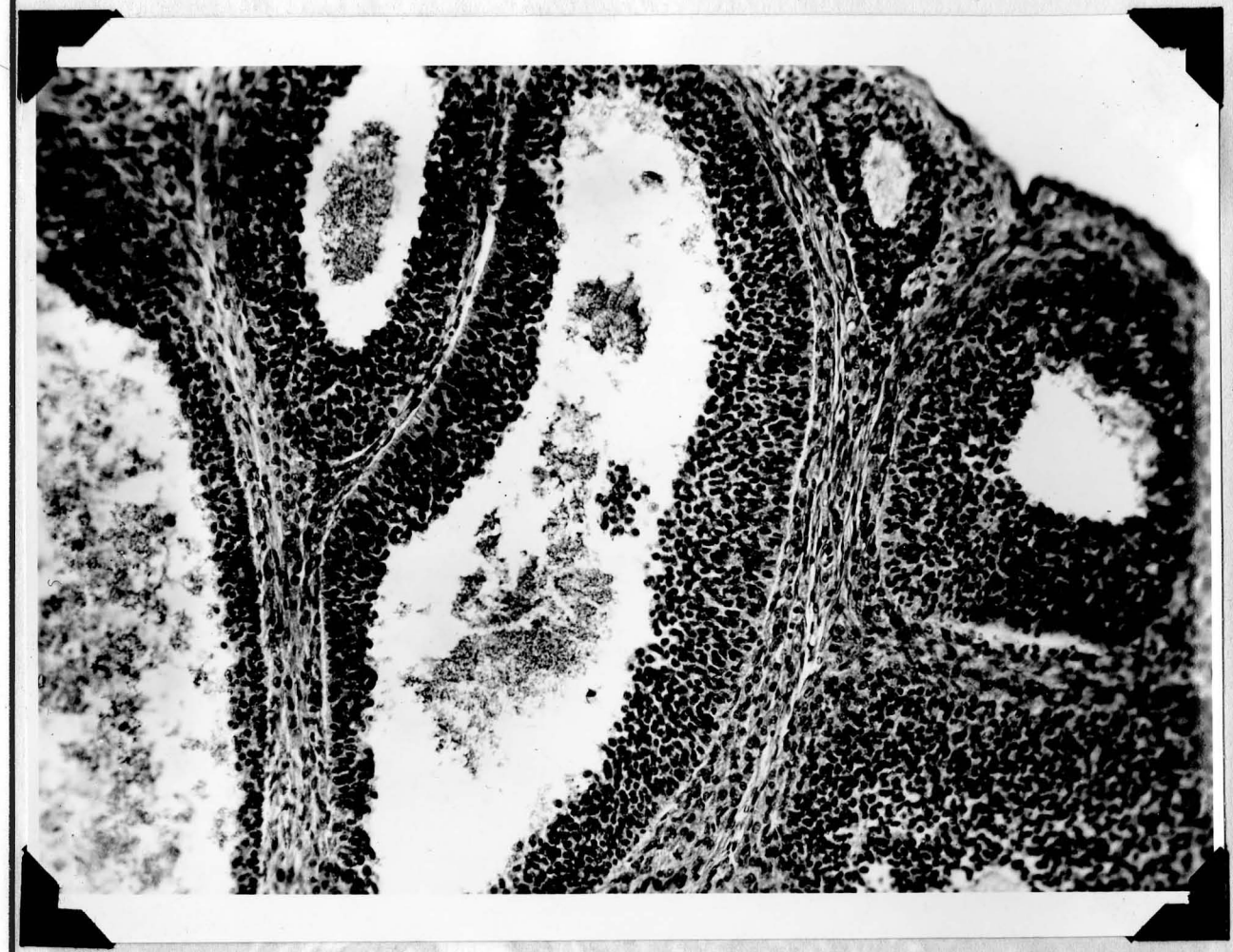
The assay animals were female hypophysectomised rats and were used six weeks post-hypophysectomy. The pituitary tissue (250 mgs) was obtained from adult female rats and fractionated in the manner described in Chapter II.

Animal No.	Dose (Mgs. N)	Body Weight (Gms.)	Weight of Organs (Mgs.) Ovaries	Uterus	Adrenals	Thyroids	Width of Epiphseal Plate
Whole Homogenate							
82	0.10	100	43	126	13	8.3	136
38	0.10	80	12	99	13	7.0	216
20	0.10	96	23	115	11	9.8	186
06	0.03	94	21	83	12	9.8	80
39	0.03	85	20	105	11	8.8	186
Nuclei							
146	0.09	85	10	85	11	7.9	114

Experiment 6 (Cont'd)

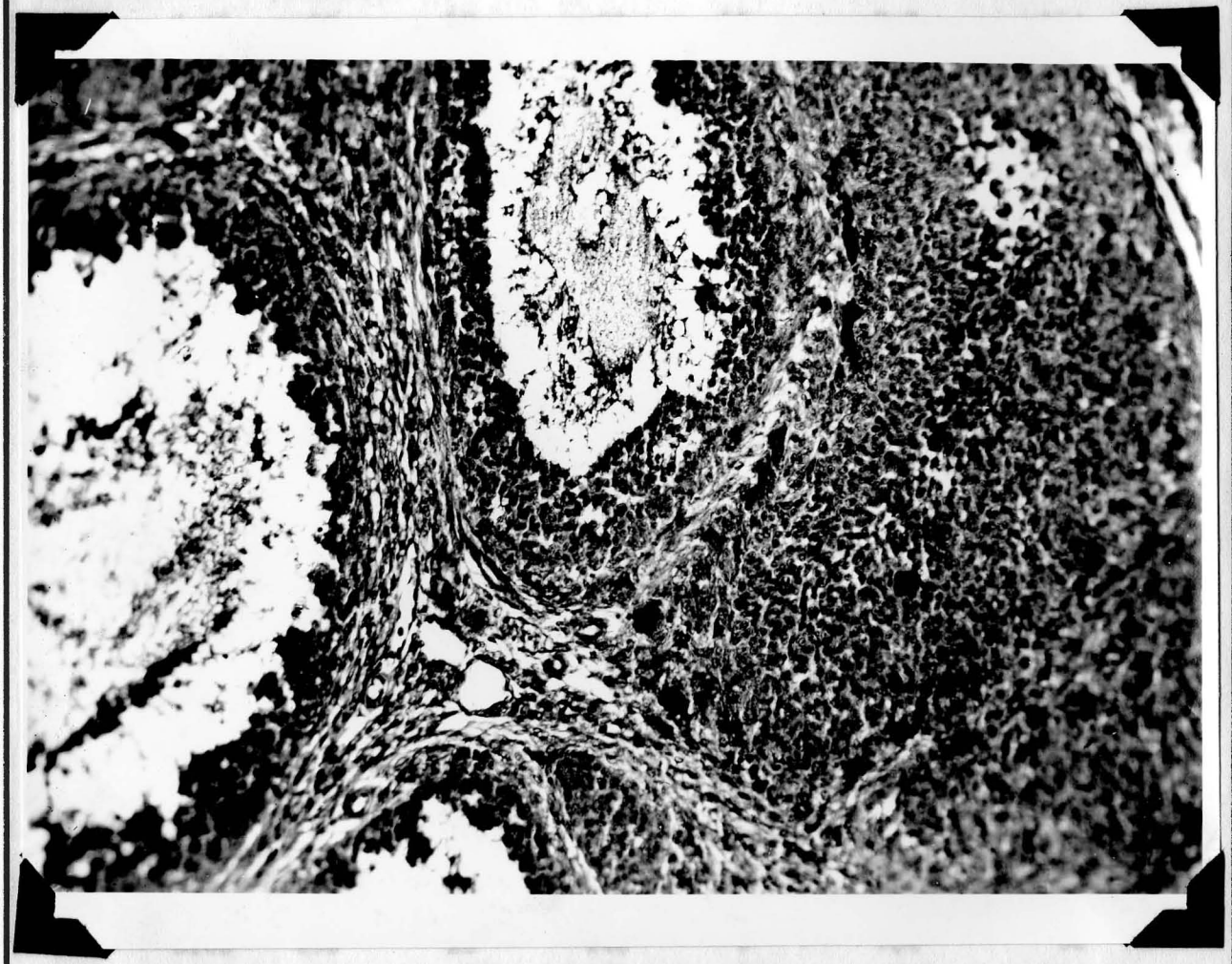
Section of ovary from control animal number 10.
Stained with hematoxylin and eosin.

Experiment 6 (Cont'd)



Section of ovary from whole homogenate animal number 41. Stained with hematoxylin and eosin.

Experiment 6 (Cont'd)



Section of ovary from large granule animal number 11.
Stained with hematoxylin and eosin.

Experiment 22 (Cont'd)

Animal No.	Dose (Mgs. N)	Body Weight (Gms.)	Weight of Organs (Mgs.) Ovaries	Uterus	Adrenals	Thyroids	Width of Epiphseal
Nuclei (Cont'd)							
26	0.09	86	13	88	10	7.7	114
73	0.09	87	14	78	13	11.4	93
25	0.03	92	12	72	11	7.3	114
94	0.03	100	8	87	12	9.0	91
141	0.03	80	12	78	9	-	74
Large Granules							
43	0.03	101	19	123	13	7.8	91
81	0.03	102	14	89	12	10.8	101
28	0.03	79	15	95	10	5.4	151
04	0.03	102	20	111	12	8.7	-
01	0.03	84	14	72	10	8.2	-
89	0.09	84	23	93	15	9.8	-
69	0.09	87	25	105	12	7.7	136
05	0.09	82	38	182	11	7.6	90
14	0.09	88	18	71	14	7.8	98
49	0.09	106	26	113	9	7.4	86
Small Granules							
242	0.09	95	-	-	-	-	-
47	0.09	86	13	43	14	7.3	190
417	0.09	80	18	38	11	8.5	214
42	0.03	92	9	69	10	6.4	85

Experiment 22 (Cont'd)

Animal No.	Dose (Mgs. N)	Body Weight (Gms.)	Weight of Organs (Mgs.) Ovaries	Uterus	Adrenals	Thyroids	Width of Epiphseal Plate
Small Granules (Cont'd)							
413	0.03	84	11	72	10	6.4	138
410	0.03	80	12	72	13	7.0	114
Supernate							
18	0.09	90	14	45	16	6.6	167
59	0.09	114	-	-	-	-	-
63	0.09	101	12	39	14	9.6	152
14	0.03	75	14	69	15	5.1	222
89	0.03	83	14	67	12	7.0	216
60	0.03	74	10	60	13	4.6	106
Controls							
97	-	77	18	80	14	7.3	67
64	-	92	-	-	-	-	91
69	-	92	13	69	11	6.1	74
74	-	91	12	-	-	-	-
23	-	91	15	81	15	9.4	74
163	-	94	15	57	11	6.0	74
21	-	80	9	7	11	8.4	80
09	-	91	12	69	12	12.0	68
610	-	103	11	20	12	6.1	72
612	-	86	13	25	13	8.3	74

Experiment 23

The assay animals were female hypophysectomized rats and were used approximately two months post-hypophysectomy. Five hundred and seventy milligrams rat pituitary tissue was fractionated in the manner described in Chapter II. In this experiment the large granule fraction was sub-fractionated.

Animal No.	Dose (Mgs. N)	Body Weight (Gms.)	Weight of Organs (Mgs.)				Width of Epiphseal Plate (Microns)
			Ovaries	Uterus	Adrenals	Thyroids	
Whole Homogenate							
25	0.15	75	6	180	15	8.5	189
55	0.15	90	13	38	13	8.1	198
415	0.15	82	14	30	13	6.0	212
20	0.30	92	5	47	15	12.0	237
56	0.30	87	6	20	11	10.3	266
31	0.30	80	37	190	17	24.0	295
846	0.60	91	15	87	14	9.9	125
72	0.60	100	28	106	12	11.8	349
Nuclei							
92	0.16	78	-	-	-	-	147
12	0.16	81	5	21	10	7.7	170
963	0.16	88	11	14	13	8.2	158
73	0.31	93	5	32	15	8.0	133
413	0.31	91	4	33	12	8.3	289
78	0.31	89	3	31	13	4.6	228

Experiment 23 (Cont'd)

Animal No.	Dose (Mgs. N)	Body Weight (Gms.)	Weight of Organs (Mgs.) Ovaries	Uterus	Adrenals	Thyroids	Width of Epiphseal Plate
Large Granules-1							
76	0.15	87	6	27	10	9.0	131
98	0.15	81	7	56	12	6.2	139
52	0.15	83	8	66	14	6.6	248
33	0.30	90	23	128	12	10.6	253
75	0.30	91	9	98	15	7.2	148
07	0.30	88	10	58	12	6.0	173
Large Granules-2							
74	0.10	81	12	30	10	9.6	193
418	0.10	81	10	38	11	12.2	167
414	0.10	87	8	30	12	12.8	261
13	0.19	85	44	262	15	13.2	292
82	0.19	95	9	92	13	10.6	170
11	0.19	91	38	186	15	8.7	301
Small Granules							
10	0.15	84	6	29	11	9.6	186
77	0.15	89	5	23	10	7.7	120
17	0.15	84	3	24	12	6.0	175
39	0.30	94	8	32	14	7.3	214
66	0.30	93	7	24	15	9.4	236
656	0.30	94	19	67	12	12.4	237
48	0.61	90	7	24	11	9.0	108
36	0.61	90	10	81	14	9.8	329

Animal No.	Dose (Mgs. N)	Body Weight (Gms.)	Weight of Organs (Mgs.)				Width of Epipseal Plate
			Ovaries	Uterus	Adrenals	Thyroids	
Supernate							
01	0.13	81	-	-	-	-	81
61	0.13	82	6	25	10	6.6	209
418	0.13	82	16	100	11	6.9	268
19	0.27	87	41	40	9	7.9	346
68	0.27	89	9	99	12	10.6	245
86	0.27	90	18	82	16	8.7	231
45	0.54	91	17	102	14	10.4	368
34	0.54	99	19	105	16	11.0	329
49	0.54	104	8	94	19	11.3	354
Controls							
30	-	92	5	31	11	7.4	117
419	-	90	4	19	12	8.6	114
21	-	87	3	19	12	6.8	102
71	-	82	4	19	14	7.8	136
412	-	86	5	23	11	8.4	72
40	-	80	5	14	14	5.4	109
Negative Controls							
	-	109	6	21	8	11.7	109
	-	92	3	24	14	9.3	112
	-	101	6	15	12	7.5	117
	-	107	5	15	14	9.3	117
	-	86	3	16	11	8.3	98

Experiment 25

The assay animals were female hypophysectomized rats and were used approximately two months post-hypophysectomy. Nine hundred and twenty milligrams of pituitary tissue was fractionated as in the previous experiments and in addition the small granule-2 fraction was obtained by subjecting the supernate to a force of 110,000 x g for 30 minutes. Only the small granule fractions and the supernate fraction were assayed in this experiment.

Animal No.	Dose (Mgs. N)	Body Weight (Gms.)	Weight of Organs (Mgs.) Ovaries	Adrenals	Thyroids	Width of Epiphseal Plate (Microns)
Whole Homogenate						
40	0.25	90	9	12	8.4	121
01	0.25	93	5	6	7.1	84
38	0.25	89	7	12	8.3	102
12	0.61	106	15	10	12.3	133
19	0.61	109	5	12	11.0	221
14	0.61	96	7	12	12.7	258
Small Granules-1						
61	0.22	80	29	14	12.8	85
94	0.22	86	11	12	11.3	81
20	0.22	90	11	13	11.6	134
29	0.56	90	12	14	7.8	116
16	0.56	97	10	12	10.0	113
06	0.56	96	8	16	8.0	186

Experiment 25 (Cont'd)

Animal No.	Dose (Mgs. N)	Body Weight (Gms.)	Weight of Organs (Mgs.) Ovaries	Adrenals	Thyroids	Width of Epiphseal Plate
Small Granules-2						
77	0.07	100	7	14	8.6	130
59	0.07	98	6	14	8.0	100
97	0.07	85	5	11	5.8	74
48	0.07	100	8	12	10.0	75
Supernate						
76	0.22	87	6	14	7.8	106
13	0.22	99	5	14	5.6	136
46	0.22	81	6	11	7.3	121
73	0.62	99	6	13	14.9	206
07	0.62	96	8	11	9.0	84
78	0.62	85	7	12	11.0	122
Controls						
74	-	95	5	11	8.1	64
71	-	81	5	9	6.4	79
49	-	81	4	9	9.1	60
51	-	92	3	6	8.1	61
16	-	96	4	10	7.2	60
32	-	97	4	9	7.3	76
31	-	93	5	8	6.9	66

Experiment 27

The assay animals were male hypophysectomized rats and were used two weeks post-hypophysectomy. Pituitary tissue from adult female rats was pooled (total weight pituitary tissue was 740 mgs.) and fractionated. The supernate fraction was divided into two parts and one portion was assayed without further treatment (supernate-1). The remaining material was adjusted to pH 5.4 with 0.2 M acetic acid and centrifuged at 24,000 x g for one hour. The sediment was designated supernate-2 and the soluble material supernate-3.

Animal No.	Dose (Mgs. N)	Body Weight (Gms.)	Weight of Organs (Mgs.) Seminal Vesicles	Testes	Adrenals	Width of Epiphseal Plate (Microns)
Whole Homogenate						
12	0.19	53	13.2	270	13	410
40	0.19	55	15.3	193	10	502
02	0.19	51	21.2	187	11	321
30	0.48	58	14.0	209	14	362
20	0.48	60	14.2	276	8	387
35	0.48	61	14.1	204	-	431
Large Granules						
60	0.16	52	12.5	290	8	322
14	0.16	54	15.1	170	10	343
11	0.16	54	20.3	230	9	327
50	0.16	54	12.2	210	10	400
15	0.40	59	18.2	290	13	-
03	0.40	61	19.1	320	10	311

Experiment 27 (Cont'd)

Animal No.	Dose (Mgs. N.)	Body Weight (Gms.)	Weight of Organs (Mgs.) Seminal Vesicles	Testes	Adrenals	Width of Epiphseal Plate
Large Granules (Cont'd)						
53	0.40	57	13.0	300	12	370
123	0.40	56	15.7	240	13	367
Small Granules						
01	0.22	55	11.0	160	13	243
00	0.22	59	11.4	250	11	341
06	0.22	52	6.7	155	10	325
04	0.22	57	-	188	-	238
13	0.56	59	14.2	220	13	373
10	0.56	57	10.2	220	12	458
36	0.56	69	-	-	20	381
72	0.56	55	8.7	202	16	335
Supernate-1						
25	0.20	52	9.8	155	12	299
26	0.20	56	8.4	195	11	429
31	0.20	52	10.5	188	10	339
43	0.20	51	15.0	185	11	381
71	0.48	55	12.6	210	13	424
41	0.48	57	11.4	193	12	405
32	0.48	62	14.0	208	10	393
70	0.48	57	14.3	203	9	361

Experiment 27 (Cont'd)

Animal No.	Dose (Mgs. N)	Body Weight (Gms.)	Weight of Organs (Mgs.)			Width of Epiphseal Plate
			Seminal Vesicles	Testes	Adrenals	
Supernate-2						
55	0.16	54	8.0	140	12	324
52	0.16	52	10.4	165	-	299
23	0.16	54	9.4	157	13	324
24	0.37	60	11.0	190	10	378
34	0.37	58	11.3	166	9	267
Supernate-3						
33	0.14	51	9.2	119	9	281
44	0.14	53	9.8	162	8	285
46	0.14	53	8.3	168	7	285
21	0.36	62	10.1	192	10	238
51	0.36	55	7.4	169	8	320
45	0.36	59	9.8	168	8	300
Controls						
05	-	51	6.5	135	8	179
62	-	57	7.0	119	9	203
63	-	53	7.6	123	11	257
82	-	56	9.6	124	9	200
68	-	57	7.3	100	10	154
08	-	55	6.3	123	10	236
66	-	55	9.4	-	-	-

Counting Data

The data from experiment 40 will be given in detail to serve as an example of the calculations involved.

Experiment 40

One hour Incubation of Pituitary Tissue

Fraction	C/M	Average C/M	C/M - Back Ground	Weight of Material in Planocts	Percent* Correction	Corrected C/M
Nuclei	377 413 363	372	292	38.1	74	395
Large Granules	474 459	467	389	32.8	76	509
Small Granules	537 585 544	555	475	34.1	76	625
Supernates	932 941	937	857	41.4	73	1174
Medium	282 266	274	194	36.2	73	259

*See Melchior and Goldkamp (1954) for self adsorption corrections.

Counted on 10-29-54

Wet weight of pituitary tissue was 143 mgs.and the labeled Methionine was batch number L-M-5.

The radioactivity of each fraction was corrected for the material removed for the nitrogen determinations which in this experiment and in most of the others was one-tenth of the TCA insoluble protein of the fractions.

Experiment 40 (Cont'd)

Fraction	Total Nitrogen	Percent Nitrogen	Percent Label	Micromols Methionine-S^{35} / Gram Protein Nitrogen
Nuclei	0.96 mgs.	24.2	13.3	2.98
Large Granules	0.52	13.1	17.2	6.12
Small Granules	0.68	18.0	21.1	6.68
Supernates	1.26	31.0	39.6	7.12
Medium	0.54	13.6	8.8	3.49

The values in the last column were calculated with the aid of the following equation:

$$\frac{(C/M)\ (f)\ (10^{-2})}{(S)\ (Mgs.\ N.)} = \frac{\text{Micromols Methionine-}S^{35}}{\text{Gram Protein Nitrogen}}$$

Where:

C/M = the total counts per minute corrected for self adsorption and for the material removed for the nitrogen determination

S = counts per minute per micromol methionine x 10^{-5} on the day the labeled amino acid was standardized. The values for the different batches of methionine were as follows: L-M-4 4.20 (5-18-53), L-M-5 4.13 (6-25-54), L-M-6 7.18 (3-4-55).

f = antilog (3.46 x 10^{-3} x days after day methionine was standardized)

To calculate the micromols methionine per gram tissue the above equation was used except that mgs. tissue was substituted for mgs. nitrogen. The total label incorported by the whole tissue is given by the following calculation:

$$\frac{(3290)\ (2.705)\ (10^{-2})}{(4.13)\ (148)} = 0.16 = \frac{\text{Micromols Label}}{\text{Gm. Tissue}}$$

Experiment 28

Zero Time Incorporation of Pituitary Tissue

Fraction	Total Nitrogen	Percent Nitrogen	C/M	Percent Label	Micromols Label / Gm. Protein Nitrogen
Nuclei	0.96	23.1	18	9.6	0.047
Large Granules-1	0.45	10.8	7	3.7	0.059
Large Granules-2	0.39	9.4	0	0.0	0.00
Small Granules	0.85	20.5	0	0.0	0.00
Supernates	1.14	27.5	6	6.9	0.029
Medium	0.36	8.6	149	80.0	1.08

Wet weight pituitary = 181 mgs.
f = 1.05, methionine number L-M-5
Micromols label/ gm. N. = 0.0036

Experiment 29

Zero Time Incorporation of Pituitary Tissue

Fraction	Total Nitrogen	Percent Nitrogen	C/M	Percent Label	Micromols Label / Gm. Protein Nitrogen
Nuclei	0.78	20.4	21	8.4	0.073
Large Granules	0.95	24.9	9	3.6	0.024
Small Granules	0.67	17.6	0	0.0	0.00
Supernates	1.03	27.0	18	7.1	0.047
Medium	0.38	10.0	204	80.9	1.46

Wet weight tissue = 98 mgs. , methionine L-M-5, f = 1.117
Micromols label/ gm. tissue = 0.007

Experiment 30

Zero Time Incorporation of Pituitary Tissue

Fraction	Total Nitrogen (Mgs)	Percent Nitrogen	C/M	Percent Label	Micromols Label / Gm. Protein Nitrogen
Nuclei	0.80	15.2	9	2.0	0.033
Large Granules	1.64	29.3	8	1.9	0.012
Small Granules	0.83	15.8	4	0.8	0.014
Supernates	1.35	26.3	23	5.0	0.480
Medium	0.70	13.3	362	89.1	1.51

Wet weight pituitary tissue = 142 mgs.
f = 1.21, methionine number L-M-5

Experiment 7

Fifteen Minute Incubation of Pituitary Tissue

Fraction	Total Nitrogen (Mgs)	Percent Nitrogen	C/M	Percent Label	Micromols Label / Gm. Protein Nitrogen
Nuclei	1.16	23.6	233	11.0	0.556
Large Granules	0.83	16.9	227	10.7	0.757
Small Granules	0.86	17.3	604	28.5	1.96
Supernates	1.45	29.4	944	44.5	1.80
Medium	0.63	12.8	109	5.1	0.479

Wet weight pituitary tissue = 118 mgs.
f = 1.19, methionine number L-M-4

Experiment 9

One Hour Incubation of Pituitary Tissue

Fraction	Total Nitrogen (Mgs)	Percent Nitrogen	C/M	Percent Label	Micromols Label / Gm. Protein Nitrogen
Nuclei	0.63	17.4	357	8.0	1.99
Large Granules	0.646	19.9	214	5.2	1.21
Small Granules	0.71	19.4	1431	34.4	7.16
Supernates	1.27	34.8	1914	46.1	5.32
Medium	0.30	8.2	239	5.7	2.01

Wet weight pituitary tissue = 115 mgs.
Methionine number L-M-4

Experiment 11

One Hour Incubation of Pituitary Tissue

Fraction	Total Nitrogen	Percent Nitrogen	C/M	Percent Label	Micromols Label / Gm. Protein Nitrogen
Nuclei	0.55	16.9	351	9.6	2.38
Large Granules-1	0.40	12.3	418	11.4	3.93
Large Granules-2	0.40	12.3	287	7.8	2.68
Small Granules	0.63	19.2	784	21.4	4.68
Supernates	0.92	28.4	1568	42.9	6.35
Medium	0.35	10.8	249	6.8	2.65

Wet weight pituitary tissue = 88 mgs.
f = 1.569, methionine number L-M-4
Micromols label / gm. tissue = 0.155

Experiment 14

One Hour Incubation of Pituitary Tissue

Fraction	Total Nitrogen (Mgs)	Percent Nitrogen	C/M	Percent Label	Micromols Label / Gm. Protein Nitrogen
Nuclei	0.97	21.4	473	15.0	2.08
Large Granules-1	0.46	10.1	289	9.2	2.63
Large Granules-2	0.44	9.8	293	9.3	2.82
Small Granules	0.85	18.9	801	25.5	3.99
Supernates	1.14	25.5	1186	37.6	4.40
Medium	0.64	12.0	106	3.3	0.70

Wet weight pituitary tissue = 142 mgs.
f = 1.779, methionine number L-M-4

Experiment 21

One Hour Incubation of Pituitary Tissue

Fraction	Total Nitrogen	Percent Nitrogen	C/M	Percent Label	Micromols Label / Gm. Protein Nitrogen
Nuclei	0.37	17.2	74	8.4	1.49
Large Granules-1	0.27	12.4	100	11.3	2.79
Large Granules-2	0.25	11.6	114	6.8	1.53
Small Granules	0.37	17.6	190	21.6	3.76
Supernates	0.63	29.6	357	40.6	4.39
Medium	0.25	11.6	108	12.3	3.19

Wet weight pituitary tissue = 45.7 mgs.
f = 3.104, methionine number L-M-4

Experiment 16

Two Hour Incubation of Pituitary Tissue

Fraction	Total Nitrogen (Mgs)	Percent Nitrogen	C/M	Percent Label	Microsols Label / Gm. Protein Nitrogen
Nuclei	0.59	16.0	371	10.5	2.94
Large Granules-1	0.50	13.6	502	14.2	4.55
Large Granules-2	0.38	10.2	179	5.1	2.12
Small Granules	0.77	20.8	842	23.9	4.94
Supernates	1.04	28.1	1460	41.4	6.34
Medium	0.42	11.3	176	4.9	1.90

Wet weight pituitary tissue = 86 mgs.
f = 1.896, methionine number L-M-4

Experiment 17

Two Hour Incubation of Pituitary Tissue

Fraction	Total Nitrogen	Percent Nitrogen	C/M	Percent Label	Microsols Label / Gm. Protein Nitrogen
Nuclei	0.45	19.7	392	12.0	4.33
Large Granules-1	0.31	13.3	409	12.5	6.55
Large Granules-2	0.19	8.2	280	8.6	7.28
Small Granules	0.41	17.9	767	23.5	9.12
Supernates	0.64	28.0	1243	38.1	9.48
Medium	0.29	12.6	173	5.3	2.89

Wet weight pituitary tissue = 72 mgs.
f = 2.052; methionine number L-M-4

Experiment 18

Two Hour Incubation of Pituitary Tissue

Fraction	Total Nitrogen (Mgs)	Percent Nitrogen	C/M	Percent Label	Micromols Label / Gm. Protein Nitrogen
Nuclei	0.46	19.7	450	13.5	5.16
Large Granules-1	0.23	10.4	344	10.3	7.46
Large Granules-2	0.23	10.	275	8.2	6.33
Small Granules	0.48	20.8	698	20.9	7.56
Supernates	0.64	28.0	1293	37.2	10.00
Medium	0.26	11.2	342	9.9	6.58

Wet weight pituitary tissue = 68 mgs.
f = 2.101; methionine number L-M-4

Experiment 26

Two Hour Incubation of Pituitary Tissue

Fraction	Total Nitrogen	Percent Nitrogen	C/M	Percent Label	Micromols Label / Gm. Protein Nitrogen
Nuclei	1.39	21.0	142	14.8	8.38
Large Granules	1.17	18.3	208	21.2	5.57
Small Granules	1.13	17.7	210	22.0	5.95
Supernate-1	1.09	17.1	268	28.1	7.87
Supernate-2	0.74	11.6	96	10.3	4.28
Medium	0.90	14.0	34	3.6	1.20

Wet weight pituitary tissue = 164 mgs.
f = 15.4; methionine number L-M-4
Supernate-1 was the material precipatated at pH 5.4
Supernate-2 was the remaining soluble protein

Experiment 16

One Hour Incubation of Liver Tissue

Fraction	Total Nitrogen (Mgs)	Percent Nitrogen	C/M	Percent Label	Micromols Label / Gm. Protein Nitrogen
Nuclei	0.67	11.4	92	8.4	0.771
Large Granules	1.08	21.8	96	9.8	0.454
Small Granules	1.189	23.9	505	46.1	2.000
Supernates	1.22	24.5	299	27.4	1.161
Medium	0.92	18.4	100	9.2	0.515

Weight of liver slices = 173 mgs.
f = 1.388, methionine number L-M-4

Experiment 16 B

One Hour Incubation of Liver Tissue

Fraction	Total Nitrogen	Percent Nitrogen	C/M	Percent Label	Micromols Label / Gm. Protein Nitrogen
Nuclei	0.72	12.7	100	8.2	0.638
Large Granules	1.51	22.4	180	12.4	0.579
Small Granules	1.21	20.8	580	47.8	2.26
Supernates	1.44	24.6	309	25.5	1.02
Medium	1.14	19.5	75	6.2	0.311

Weight of liver slices = 179 mgs.
f = 1.988, methionine number L-M-4

Experiment 19

One Hour Incubation of Liver Tissue

Fraction	Total Nitrogen (Mgs)	Percent Nitrogen	C/M	Percent Label	Microsomls Label / Gm. Protein Nitrogen
Nuclei	0.68	12.9	65	6.9	0.50
Large Granules	1.13	22.5	88	9.3	0.46
Small Granules	1.14	21.4	386	41.0	1.78
Supernates	1.15	21.6	300	31.3	1.37
Medium	1.14	21.5	103	10.9	0.47

Weight of liver slices = 194 mgs.
f = 2.205; methionine number L-M-4

Experiment 31

Two Hour Incubation of Liver Tissue

Fraction	Total Nitrogen	Percent Nitrogen	C/M	Percent Label	Microsomls Label / Gm. Protein Nitrogen
Nuclei	1.50	21.3	530	12.3	1.17
Large Granules	1.22	17.4	622	14.7	1.69
Small Granules	1.11	15.8	1417	33.4	4.23
Supernates	1.43	20.3	915	21.5	2.13
Medium	1.77	25.2	757	17.8	1.42

Weight of liver slices = 181 mgs.
f = 1.372; methionine number L-M-5

Experiment 31 B

Two Hour Incubation of Liver Tissue

Fraction	Total Nitrogen (Mgs)	Percent Nitrogen	C/M	Percent Label	Micromole Label / Gm. Protein Nitrogen
Nuclei	1.84	19.6	767	10.4	1.90
Large Granules	1.81	19.1	1265	17.1	3.20
Small Granules	1.18	17.2	2398	32.5	6.07
Supernates	1.57	22.9	2027	27.4	4.29
Medium	1.45	21.2	930	12.6	2.12

Weight of liver slices = 173 mgs.
f = 1.87%; methionine number L-M-5

Experiment 32

Two Hour Incubation of Liver Tissue

Fraction	Total Nitrogen	Percent Nitrogen	C/M	Percent Label	Micromole Label / Gm. Protein Nitrogen
Nuclei	1.08	17.7	962	8.8	3.23
Large Granules	1.14	19.6	1575	14.4	4.35
Small Granules	1.10	18.9	4555	41.9	14.56
Supernates	1.53	22.8	2464	22.6	6.49
Medium	1.32	22.7	1329	12.2	3.52

Weight of liver slices = 165 mgs.
f = 1.44%; methionine number L-M-5

Experiment 32 B

Two Hour Incubation of Liver Tissue

Fraction	Total Nitrogen (Mgs)	Percent Nitrogen	C/M	Percent Label	Micromols Label / Gm. Protein Nitrogen
Nuclei	1.13	20.7	881	8.2	2.64
Large Granules	0.94	17.2	1169	11.2	4.38
Small Granules	0.95	17.4	4482	43.0	16.63
Supernates	1.24	22.7	2664	25.5	7.82
Medium	1.20	22.0	1280	12.0	3.74

Weight of liver slices = 174 mgs.
f = 1.446; methionine number L-M-5

Labeling Data for Diethylstilbesterol Treated Animals

Experiment 48
Normal Controls

Fraction	Total Nitrogen (Mgs)	Percent Nitrogen	C/M	Percent Label	Micromols Label / Gm. Protein Nitrogen
Nuclei	0.75	23.0	112	13.1	2.10
Large Granules	0.54	16.1	147	17.2	3.82
Small Granules	0.57	17.5	160	18.7	3.94
Supernates	0.96	29.7	330	38.6	4.90
Medium	0.48	14.6	107	12.4	3.14

Wet weight pituitary tissue = 126 mgs. Average weight = 12.6
f = 5.770; Methionine number L-M-5

Experiment 49

Pituitary Tissue from Treated Animals

Fraction	Total Nitrogen (Mgs)	Percent Nitrogen	C/M	Percent Label	Micromols Label / Gm. Protein Nitrogen
Nuclei	0.04	18.4	147	9.6	3.33
Large Granules	0.59	16.9	338	22.2	8.38
Small Granules	0.88	25.4	444	29.1	7.34
Supernates	1.08	31.2	512	33.6	6.88
Medium	0.25	8.1	34	5.5	4.21

Weight pituitary tissue = 154 mgs.
Average weight = 15.4 mgs.
f = 6.006; Methionine number L-M-5

Experiment 50

Pituitary Tissue from Treated Animals

Fraction	Total Nitrogen	Percent Nitrogen	C/M	Percent Label	Micromols Label / Gm. Protein Nitrogen
Nuclei	0.35	23.6	156	12.5	2.75
Large Granules	0.53	14.7	243	19.4	6.88
Small Granules	0.71	19.7	370	29.6	7.80
Supernates	1.10	30.6	426	34.1	5.82
Medium	0.41	11.4	56	4.5	2.05

Weight pituitary tissue = 133 mgs.
Average weight = 16.6 mgs.
f = 6.198

Experiment 60 B

Pituitary tissue from Treated Animals

Fraction	Total Nitrogen (Mgs)	Percent Nitrogen	C/N	Percent Label	Micromols Label / Gm. Protein Nitrogen
Nuclei	0.85	22.0	176	14.0	3.10
Large Granules	0.56	14.6	251	16.9	6.68
Small Granules	0.76	19.7	307	24.4	6.06
Supernates	1.26	32.6	466	36.9	
Medium	0.44	11.3	55	4.8	2.12

Weight of pituitary tissue = 149 mgs.
Average weight = 12.4
f = 6.198; Methionine number L-M-6

APPROVAL SHEET

The dissertation submitted by Daniel M. Ziegler has been read and approved by five members of the faculty of the Stritch School of Medicine, Loyola University.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form, and mechanical accuracy.

The dissertaion is therefore accepted in partial fulfillment of the requirement for the Degree of Doctor of Philosophy.

May 24, 1955
Date

Jacklyn B. Melchior
Signature of Adviser